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CELLULAR CONSEQUENCES OF PRETERM BIRTH – TELOMERE BIOLOGY, IMMUNE DEVELOPMENT AND OXIDATIVE STRESS

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Cellular consequences of preterm birth – telomere biology, immune development and oxidative stress

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Till Mormor – with curiosity everything is possible

ABSTRACT

Preterm infants are at risk for oxidative stress just by being born. The extra-uterine environment is relatively oxygen-rich, their antioxidant defenses are immature, diseases and treatments in the neonatal period will trigger inflammatory responses. The cellular effects of preterm birth and the impact on immune development are incompletely understood.

The main focus of this thesis is trying to understand the cellular mechanisms of how preterm birth affect the adaptation to extrauterine life. The four papers included involve different aspects of environmental exposures in children delivered preterm; telomere length, inflammation and lung function (paper I), viral respiratory infections and cellular aging using the biological markers telomere length (telomere attrition rate) and predicted DNA methylation biological age (paper II), immune system development and environmental exposures (paper III), and hyperoxia-induced lung damage in an experimental model and the capacity to counter-act surfactant inactivation with a novel antioxidant (paper IV).

We found that telomere length was similar in 10-year-old children born preterm with a history of BPD and term born children with allergic asthma. Impaired lung function with low forced expiratory capacity and male gender were associated with short telomeres irrespectively of preterm birth (paper I). Despite early exposures to risk factors, preterm born children had preserved telomeres and showed no accelerated epigenetic aging during the first 2 years of life (paper II). Measurements of immune system states that cord blood was not representative of postnatal immunity. The immune system of preterm and term children differed at birth but unexpectedly converged early in life and followed a shared stereotypic pattern of adaptation to environmental exposures. Microbial interactions drive early immune system development (paper III). Hyperoxia impaired surfactant function and this could not be prevented by an antioxidant, N-Acetylcysteine amide, however the antioxidant did not affect surfactant function or treatment effect (paper IV).

We have developed new sampling methods allowing us to perform comprehensive measurements from minimal blood sample volumes, particularly important in preterm infants with small blood volumes. The resulting “neonate-omics” permits global assessments of immune system composition to be related to biochemical pathways and epigenetic modulations.

In conclusion, preterm birth was not associated with increased cellular aging, suggesting active repair mechanisms compensating for neonatal stressors. Exogenous surfactant is a vehicle for antioxidant treatment to the lung. We describe for the first time the immune adaptation to environmental exposures early in life. With a better understanding of the challenges for a baby born far too early and much too small comes the possibility to develop individualized treatments and modify care to ensure not just survival, but future health.

LIST OF SCIENTIFIC PAPERS

- I. **Telomere length was similar in school-age children with bronchopulmonary dysplasia and allergic asthma**
Ewa Henckel, Ulrika Svenson, Björn Nordlund, Eva Berggren-Broström, Gunilla Hedlin, Sofie Degerman and Kajsa Bohlin
Acta Paediatrica 2018; 107:1395-401. DOI: 10.1111/apa.14294
- II. **Hematopoietic cellular aging is not accelerated during the first two years of life in children born preterm**
Ewa Henckel, Mattias Landfors, Zahra Haider, Paraskievi Kosma, Magnus Hultdin, Sofie Degerman* and Kajsa Bohlin*
Manuscript
- III. **Stereotypic Immune System Development in Newborn Children**
Axel Olin*, Ewa Henckel*, Yang Chen, Tadepally Lakshmikanth, Christian Pou, Jaromir Mikes, Anna Gustafsson, Anna Karin Bernhardsson, Cheng Zhang, Kajsa Bohlin and Petter Brodin
Cell 2018; 174: 1277-92.e14. DOI: 10.1016/j.cell.2018.06.045
- IV. **Surfactant mixed with the antioxidant N-acetylcysteine amid (NACA) to prevent hyperoxia-induced impaired lung function in an experimental rabbit model**
Ewa Henckel, Marie Haegerstrand-Björkman, Svante Norgren, Tore Curstedt and Kajsa Bohlin
Manuscript

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LIST OF ABBREVIATIONS

bp	Base pairs
BPD	Bronchopulmonary dysplasia
DNA	Deoxyribonucleic acid
DNAm	DNA methylation
FDR	False-discovery rate
IGF-1	Insulin growth factor 1
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
NEC	Necrotizing enterocolitis
IL	Interleukin
IVH	Intraventricular hemorrhage
HIF	Hypoxia-inducible factor
hTERT	Human telomerase reverse transcriptase
hTERC	Human telomerase RNA gene
LBW	Low birth weight
VLBW	Very low birth weight
ELBW	Extremely low birth weight
LPS	Lipopolysaccharide
NACA	N-Acetylcysteine amide
NADPH	Nicotineamide adenine dinucleotide phosphate
NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
PC	Phosphatidyl choline
RNA	Ribonucleic acid
ROP	Retinopathy of prematurity
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
PDA	Persistent ductus arteriosus

PBMC	Peripheral blood mononuclear cell
SIRT1	Nicotinamide adenine dinucleotide dependent deacetylase
SOD	Superoxide dismutase
TLR	Toll-like receptor
TNF- α	Tumor-Necrosis Factor-Alpha
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

1 BACKGROUND

1.1 PREMATURITY

1.1.1 Definitions and prevalence

Complications of preterm birth caused ~1 million deaths in the world in 2015 and was the leading cause of death among children under five years of age to the World Health Organization ³. One in 10 children are born preterm, which is defined as birth before 37 completed weeks of gestation:

- extremely preterm: less than 28 weeks of gestation
- very preterm: 28⁺⁰ to 31⁺⁶
- late to moderate: 32⁺⁰ to 36⁺⁶

Sometimes the definitions used are based on birth weight instead ⁴:

- low birth weight (LBW): < 2500 grams
- very low birth weight (VLBW): < 1500 grams
- extremely low birth weight (ELBW): < 1000 grams

Suggestions from WHO in order to save 75% of preterm babies involve the implementation of essential care for both mother and child at birth and postnatally, provision of antenatal steroid injections to mothers at risk of preterm delivery, kangaroo mother care and antibiotics to treat newborn infections.

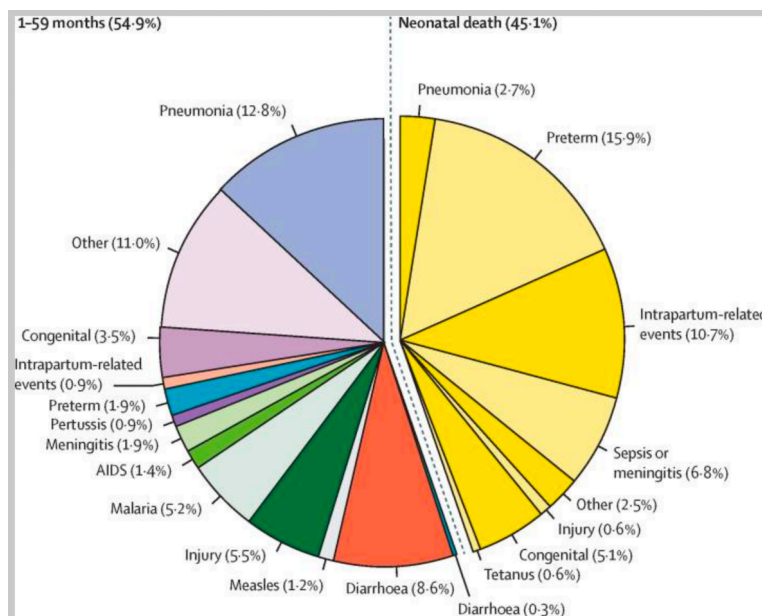


Figure 1. Global causes under-5 deaths in 2015. Source: WHO ³

Around 115 000 children are born in Sweden per year, the incidence of preterm birth is less than 6% and 1% of infants are born very preterm. A Swedish population-based study of

infants born alive, prior to 27 weeks of gestation between the years 2004 and 2007, the EXPRESS study, reported an overall one-year survival of 70% ⁵.

1.1.2 Short term lung morbidity

Immature lungs are still a major cause of mortality and morbidity in infants born extremely preterm and in the Swedish cohort 25 % developed severe bronchopulmonary dysplasia (BPD) ⁶. The use of antenatal steroids, postnatal surfactant treatment, improvements in ventilation treatments and nutritional care have ameliorated morbidity for extremely preterm born infants, but the incidence of BPD has not decreased ⁷. Definition of BPD is still based on a dependency of excess oxygen at 28 days of life and then its severity is graded at 36 weeks of gestation ⁸. Discussions are of a redefining of this disease. Previously BPD was caused by barotrauma and oxygen toxicity but today it is more considered a condition caused by an arrested normal lung growth and vascularization together with an inflammatory milieu in the preterm baby and an important influence of persistent patent ductus arteriosus (PDA) ⁹⁻¹¹. Oxidative stress plays an important role in triggering cell apoptosis, in serving as second messenger and a mediator in signal transduction. Oxidative stress can trigger cellular and molecular changes in the lung that leads to permanent changes in the lung anatomy. Many efforts have been made to prevent BPD or reduce its severity, but the lack of a clear pathophysiology is hampering such progress.

1.1.2.1 Ventilation strategy to prevent lung injury

Mechanical ventilation is known to cause structural effects on lung development in preterm infants ¹² and a meta-analysis show that more non-invasive ventilation strategies reduce BPD incidence ¹³. Mechanical ventilation, as oxidative stress, can induce inflammation with cytokines being released from macrophages and neutrophils ¹⁴. Inhaled nitric oxide (NO) could help advance lung development in animals, but NO treatment in preterm infants have not reduced incidence or mortality caused by BPD ^{15,16}. This despite some reports have shown a reduction in some inflammatory markers in tracheal aspirates after NO treatment ¹⁷.

1.1.2.2 Treatments to reduce BPD

BPD incidence have been shown to be reduced by several other treatment strategies such as caffeine ¹⁸, vitamin A injections ¹⁹ and insulin growth factor-1 (IGF-1) infusions ²⁰. We know that trace elements serve as important co-factors of antioxidants and improved nutritional status will improve the balance between catabolism and anabolism in the preterm baby and reduce BPD incidence as well as another important complication, retinopathy of prematurity (ROP) ²¹. The pathophysiology of BPD is multifactorial and clearly there is still not “one treatment” that will prevent all cases of BPD.

1.1.3 Long term morbidity

Prematurity is emerging as a risk factor for morbidity also in adult life and the risk for elevated mortality is ~ 40% ^{22,23}. There is a growing body of evidence for morbidity suggesting increased incidence of cardiovascular diseases ²⁴⁻²⁷ as well as metabolic diseases

²⁸⁻³⁰. The overall physical performance is reduced and correlates with gestational age, preterm birth and low birth weight ^{31,32}.

Respiratory conditions in childhood as well as later in life during adulthood, in children with BPD, involve airway obstruction, hyper-responsiveness and hyperinflation ³³⁻³⁵. Children born in the last two decades had better lung function as a reflection of better neonatal care but still show signs of obstruction, particularly in the small airways, and a hyper-responsiveness ³⁶⁻⁴⁰. Impaired lung function was still evident as a consequence of extremely preterm birth even without being diagnosed with BPD ⁴¹⁻⁴³.

1.2 ADAPTATION TO EXTRAUTERINE LIFE

The stress of being born is greater than any other challenge we meet in life. Transition from fetal life means adjustment of circulation, regulation of body temperature, metabolic changes and start of respiration. The fluid filled lungs will by the first few breaths need to be a sufficient provider of oxygen and remove carbon dioxide to ensure adequate ventilation. The gas exchange take place in the smallest part of the lung, the alveolus, and for its optimal function and stabilization the alveoli needs surfactant. When the lung opens up, the pulmonary resistance falls and pulmonary circulation increases. The main purpose for the blood circulation is to deliver nutrients and oxygen to all cells and remove carbon dioxide and other waste products. For an optimal oxygenation of all cells in the body the collaboration between the lung tissue and the blood flow is essential.

1.2.1 Facing the environment

The lung, the gastrointestinal tract and the skin are our interfaces with the world surrounding us and these interfaces are constantly exposed to the environment. These tissues, the lung epithelium, the gut mucosa and the epithelial layers of the skin all serve as barriers to protect us and interact with the environment. A well-adapted such environmental interaction is necessary for survival. This environmental interaction will occur irrespectively if born preterm or term, and therefore immaturity of these systems will affect the ability of a child to interact appropriately. It can be harmful but also serves as an opportunity for us as physicians to intervene. We still do not fully understand how the micro-environment affect our cellular responses and trigger our surveillance mechanisms and the responses of our immune system.

1.2.1.1 Lung

The lung matures during fetal life and a preterm lung consists of 150 million alveoli with a surface of around 3-5 m². An adult has a lung surface of around 80-100 m² and 300 million alveoli ⁴⁴. When the extremely preterm born infant meets the environment the lung development has reached a late canalicular stage or early saccular stage and the development of the alveoli is incomplete. Alveolarization is the process of forming new alveoli and normally occur postnatally like the development of the pulmonary vascular tree, vascularization. The capillaries develop and grow around the alveoli. This process is regulated by hormones and cytokines important for cell growth and differentiation. The

respiratory epithelium will be exposed to the environment and disturbances of both alveolarization and vascularization can be part of the development of BPD and pulmonary hypertension ^{45,46}.

The airways are covered with an epithelium that produce mucus, about 20 ml per day (in adults). This is the first line of defense against microbes removing pathogens and debris by the ciliary escalator. The sodium and chloride content are about the same as in plasma and pH acidic (6.8). The epithelial lining fluid contain antioxidants (glutathione, ascorbic acid, uric acid) and other biochemicals that together with macrophages and neutrophils are part of the innate immunity that protects us from the environment.

1.2.1.2 Pulmonary surfactant

The pulmonary surfactant is essential for the adaptation of the lung and for respiration. Surfactant will stabilize the alveoli during respiration by lowering the high surface tension of the air-liquid interface and without this gas exchange will be insufficient. Exogenous installed surfactant in preterm infants with respiratory distress syndrome is life-saving ⁴⁷. Surfactant also have important functions for innate immunity. Surfactant is produced by type II alveolar cells and consist of 90% lipids and 10% proteins and are essential for decreasing the surface tension and stabilize the alveoli during respiration, especially surfactant protein B and C ⁴⁸. The surface activity of surfactant depends on gestational age and the phosphatidylcholine content ⁴⁹.

The antioxidant capacity of surfactant increases with greater phospholipid content of which plasmalogens and polyunsaturated phospholipids are particularly important ⁵⁰. Activity of the two important free radical scavengers superoxide dismutase and catalase is present in surfactant. These scavengers were also found being active in naturally derived surfactant used for exogenous surfactant treatment ⁵¹.

Surfactant proteins also have properties important for immunity. Surfactant protein A and D are so called collectins and will bind to sugars on the surface of pathogens to facilitate phagocytosis and inhibit proliferation as part of the innate immune system ^{52,53}. SP-A have an important role in reducing type II alveolar cell apoptosis ⁵⁴. SP-D also have an anti-inflammatory effect binding directly to toll-like receptors (TLRs) involved in the innate immunity. The lipid content is 85% phosphatidyl choline (PC) of which the major part is saturated in the dipalmitoylated form (DPPC). The rest of the lipid content are phosphatidylglycerol, cholesterol and free fatty acids. Lipids have function in the immune system ^{55,56} and hyperoxia reduces phospholipid production of surfactant ^{57,58}.

1.2.1.3 Gastrointestinal tract

The surface area of the gastrointestinal tract in adults is around 30-40 m² and serve as another large interface with the environment along with the lung ⁵⁹. The mucosa is filled with blood and lymphatic capillaries embedded in the connective tissue covered by an epithelial lining. The gut-barrier will absorb nutrients and promote passage of molecules and signaling

molecules important in maintaining the homeostasis of endocrinal, neuronal and immunological processes. Acidity in the stomach serve as one barrier of protection against microbes along with a production of secreted factors covering the mucosal lining amounting to and around 7 liters a day (adults) that will pass through the gastrointestinal tract ⁶⁰.

1.2.1.4 Micro-environment

In late stages of gestation a rapid elevation in activity (>150%) in the antioxidant enzymes occur, much like the increases seen in the surfactant system as it prepares for the transition from the intrauterine environment to extrauterine life ⁶¹. These adaptations are an evolutionary advantage when the newborn child meets normoxia after birth in contrast to the “physioxia” during fetal life. Every tissue has its own optimal partial pressure of oxygen, “physioxia” ⁶², that during fetal life represent a much lower partial oxygen pressure than in air. Oxygen level in air is 21% (partial pressure of 160 mmHg) and in the body it differs from zero to 19%. Oxygen level is almost 0% in the lumen of the gastrointestinal tract ⁶³ where many commensal anaerobic bacteria constitutes the microbiome. In the airways oxygen level normally have around 19% of oxygen in the upper airways down to levels around 15% (110 mmHg) in the alveoli. In venous blood pO₂ is 40 mmHg (5%) and in arterial blood 100 mmHg (13%) normally. At birth the infant will transition from hypoxia to normoxia, that can be described as a hyperoxic challenge.

The cells lining the mucosa and the respiratory epithelium have the ability to attract macrophages, chemokines and work with signaling both between and within cells. Processes of attracting immune cells, phagocytosis, opsonizing, both active and passive transportations through cell membranes are at work. When the local environment change, the repertoire of responses to the environment changes and one example of that is different reactions to oxygen.

Surfactant is synthesized by type II alveolar cells already in utero and the lungs reach sufficient surfactant pools around gestational week 32-34. Born prior to that will cause surfactant deficiency, but within a couple of days after birth, the surfactant production is drastically enhanced. Term infants have a surfactant pool of 100 mg/kg while preterm have around 5 mg/kg. The lamellar bodies recycle most of the surfactant from the alveolar space back to the pneumocytes. Half-life is around 10-15 hours. Through exocytosis (transporting out from the cell) the lamellar bodies carry surfactant that form a tubular myelin net when excreted on the surface of the air-liquid phase.

1.2.2 Exposure to oxygen

1.2.2.1 Reactive oxygen species

Oxygen is necessary for survival, but it can also be toxic. Reactive oxygen species (ROS) are important for many cellular mechanisms including intracellular signaling, growth and organ development but are also important for the immune system and our defense against

microbes ⁶⁴. However, an excess of ROS can be harmful and cause DNA damage, lipid peroxidation and protein degradation (Figure 2).

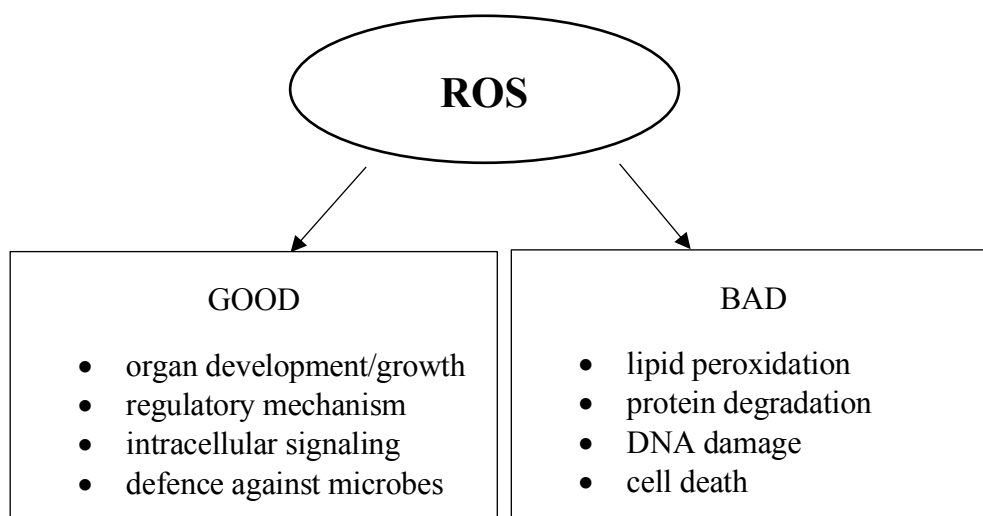
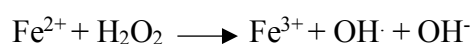


Figure 2. Reactive oxygen species are essential to life, but can also be toxic. If the antioxidant capacity is overwhelmed by production of ROS, oxidative stress will occur, the unbalance between pro-and antioxidants.

The production of ROS occurs normally in the mitochondrial respiratory chain when oxygen is reduced to water and in the process form the oxygen radical superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^{\cdot}). Oxygen radicals are also produced in different enzymatic systems of which the hypoxanthine-xanthine oxidase system might be of particularly importance in newborn infants ⁶⁵. The activation of neutrophils and macrophages is also associated with the release of mediators such as lysozymes, peroxidases, proteases, as well as oxygen radicals and nitric oxide as a mode of defense against pathogenic bacteria. Ceruloplasmin and transferrin are potent antioxidants, but when iron is oxidized (Fe^{2+} to Fe^{3+}) the most potent oxidant in the biological system, the hydroxyl radical, is formed through the Fenton reaction ⁶⁶:



The antioxidant system can under normal conditions counter-act normal ROS production, but when there is an imbalance between ROS production and the capacity of the antioxidant system we get oxidative stress.

1.2.2.2 Antioxidant defense

A compound that can donate a single uncoupled electron to a free radical will have an antioxidant capacity and be part of the defense against free radicals. Some antioxidants are produced during normal metabolism in the body but some needs to be supplemented by our diet. Vitamin A, C and E, beta-carotene as well as bilirubin and selenium are important non-enzymatic antioxidants and half of the antioxidant capacity in human blood comes from uric

acid. Enzymatic scavengers like superoxide dismutase (SOD), catalase and glutathione peroxidase play a major role in the intracellular defense.

Glutathione (GSH) is the most important intracellular antioxidant and consists of the amino acid glutamate, cysteine and glycine. Glutathione peroxidase (GPx) catalyzes the reaction converting hydrogen peroxide to water and molecular oxygen, a reaction otherwise mainly driven by catalase. In the same step GSH is oxidized to GSSG (glutathione disulfide). The oxidized form of glutathione (GSSG) will be reduced by NADPH (nicotinamide adenine dinucleotide phosphate) back to GSH (Figure 3) and the ratio of GSH/GSSG is a used marker of oxidative stress.



Figure 3. The oxidation-reduction pathway of glutathione. Glutathione peroxidase (GPx) oxidize glutathione to form GSSG and with the presence of NADPH glutathione reductase (GR) will catalyze the reaction to regenerate GSH. Glutathione is kept mainly in its reduced form in erythrocytes important for cell membrane stability and protection against ROS-induced damage.

1.2.2.3 Oxidative stress

Oxygen toxicity leads to DNA damage and even brief periods of excess oxygen exposure at birth have been associated with childhood cancer^{67,68}. On the other hand, Northway et al⁶⁹ showed in a newborn mouse model that inhibition of DNA synthesis by 100% oxygen exposure had a major effect on lung growth and cell replication but was reversal in contrast to adult mice of whom all died. This imply an adaptive capacity for cellular survival in the immature lung. Solberg et al showed in a piglet model of hypoxia that resuscitation with oxygen (40%, 60% and 100%) would in a dose-dependent manner increase oxidative stress and oxidation of DNA⁷⁰ and that hyperoxia may lead to genetic instability. Another reason for different responses to oxidative stress might be reduced antioxidant capacity due to lack of substrates, for instance cysteine, but treatment with infusions of amino acids the first days of life could not reduce oxidative stress even though levels of glutathione were increased⁷¹. Neonatal animal might resist mortality to oxygen exposure but the lung will be damaged and normal lung development changed into a phenotype similar to BPD, as recently described in an overview by Silva et al⁴⁵.

Hyperoxia leads to cell destruction, edema and inflammation. The different response to hyperoxia in an adult tissue compared to a neonatal or preterm tissue suggests different ways of signaling in the cells. Either cell death by necrosis/apoptosis or cell survival, but then often with consequences of structural changes or inflammation. That means to an altered development of the neonatal preterm lung, leading to long term consequences, explaining some of the pathophysiology of bronchopulmonary dysplasia.

1.2.2.4 *Oxidative-stress induced morbidity*

Preterm infants are at risk for oxidative stress just by being born. The extra-uterine environment is comparable rich in oxygen (P_{O_2} 100 mmHg) compared to intra-uterine life (20 mmHg). They are often exposed to chorioamnionitis and inflammation already in fetal life and need treatment with supplementary oxygen causing hyperoxia after birth. Increased ROS production due to elevated levels of free iron and the immaturity to respond to the oxidative stress⁷² will put the preterm infant at high risk for free-radical induced damage and inflammation⁷³. Also, the fact that preterm birth also occurs as a consequence of inflammatory and infectious diseases in the mother, increases the risk of elevated ROS in preterm children. The free radical disease in the neonatal period was first established by Saugstad in 1988⁶⁵. Even though the pathophysiology still is not fully understood, oxidative stress will contribute to the diseases of prematurity such as BPD, ROP, NEC, IVH and PDA⁷⁴⁻⁷⁷.

Children born preterm, irrespectively of BPD diagnosis or not, had signs of oxidative stress along with impaired lung function at 15 years of age when compared to healthy controls⁷⁸. Consequences of resuscitation or treatment with supplementary oxygen showed oxidative stress measured as different aspects of the glutathione metabolism⁷⁹⁻⁸³.

1.2.3 **Innate immune system in neonates**

The first lines of defense meeting the environment are cells of the innate immune system. This aspect of immunity is ready to go immediately at birth and does not require memory formation and interaction with specific antigens to evolve. The immunological barrier can be described as mechanical, chemical and microbiological. The mechanical defenses consists of the epithelia cells with the flow of air and fluid across the epithelium and the movement of mucus by ciliary function. The chemical defense consists of fatty acids, enzymes, low pH and antibacterial peptides. The microbiological defense here means having a non-pathogenic normal flora of microbes that will compete with the pathogens for survival. For instance, the microbiome can be called “an organ within the organ” because it can execute enzymatic reactions that the body itself cannot catalyze⁸⁴.

Another key defense strategy of the innate immune system is phagocytosis. Lung resident macrophages are the first to engulf the pathogens, but neutrophils will also help out. These cells will release granules containing enzymes and peptides that will be part in mediating this very intricate antibacterial response moving from extracellular compartment to intracellular action (Figure 4).

The immune responses can differ depending on the micro-environment, that is, which compartment or tissue involved, the oxygen level, substrates available and also maturation of the organ. The cascades of reaction can differ during developmental stages in life, the fetus will react differently compared to newborn infants and adults. The reactions in extremely preterm born infants are therefore difficult to predict. Some important factors involved in the preterm innate immune system are listed below.

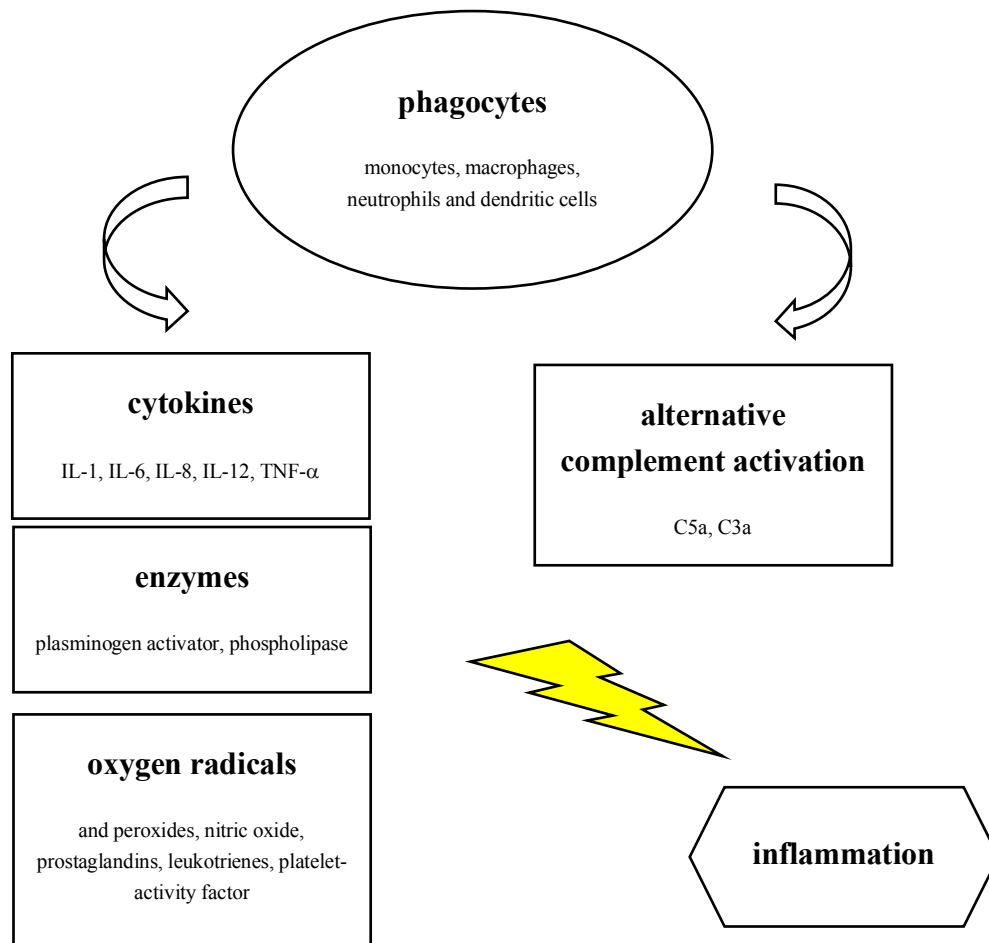


Figure 4. Phagocytosis is a key effector mechanism of the innate immune system.

1.2.3.1 Hypoxia inducible factor (HIF)

Hypoxia-inducible factor (HIF) is a protein complex with two subunits α and β that regulates transcription of over 100 genes important for regulation of cellular stress and metabolism sensitive to different oxygen levels⁸⁵. During physiological oxygen levels (normoxia) HIFs are hydroxylated by proline hydroxylases and constantly degraded. Hypoxia leads to translocation of HIF-1 α to the nucleus and together with HIF-1 β they form a complex with DNA to regulate transcription of genes involved in many different processes (angiogenesis, erythropoiesis, apoptosis, glucose metabolism, pH regulation, proteolysis, cell proliferation and survival), all of which are important during fetal life and for organ development. Hypoxia inducible factor is the key regulator of homeostasis for cell survival in a poorly oxygenated environment.

HIF-1 α accumulation activates vascular endothelial growth factor (VEGF) expression promoting the angiogenesis and the vasculogenesis^{46,86}. Hyperoxia to preterm lambs caused a dramatic decrease in levels of HIF-1 α and HIF-2 α and disrupted VEGF expression implicating that disrupted HIF and VEGF expression in the lung may contribute to BPD⁸⁷.

The micro-environment of an infected tissue is often hypoxic. The HIF levels in macrophages and neutrophils will increase when the cells encounter the more hypoxic tissue than when

circulating in well-oxygenated blood. Pathogens invading the tissue will activate TLRs that recognize shared microbial components, such as lipopolysaccharide, LPS, on gram-negative bacteria. This TLR activation will often trigger the upregulation of HIF via the NFκB pathway. These bactericidal and proinflammatory processes are enhanced and apoptosis inhibited in favor of cell survival. HIF will thereby promote the phagocytotic capacity in tissue of the infection, but not in healthy tissue where it could cause damage to the host cell. HIF also regulate other intrinsic immune responses and will enhance functions of immune cells such as dendritic cells, mast cells and epithelial cells in the skin, gut and respiratory epithelium ⁸⁸. As another example, in response to the respiratory syncytial virus, RSV, HIF expression has been shown to be induced and VEGF production increased leading to an increased blood vessel permeability and edema central to the clinical presentation of RSV pneumonia ⁸⁹. In these ways, these key components TLRs, NFκB and HIF, form a network that intersect hypoxia, inflammation and immune defense and are of importance to many of the complications facing children born preterm.

1.3 BIOMARKERS AND ANTIOXIDANTS

1.3.1 Markers of oxidative stress

Biomarkers for oxidative stress are difficult to measure because free radicals have a very short half-life. Instead more stable products of the free radical damage on lipids, proteins and nucleic acids are measured (Figure 5).

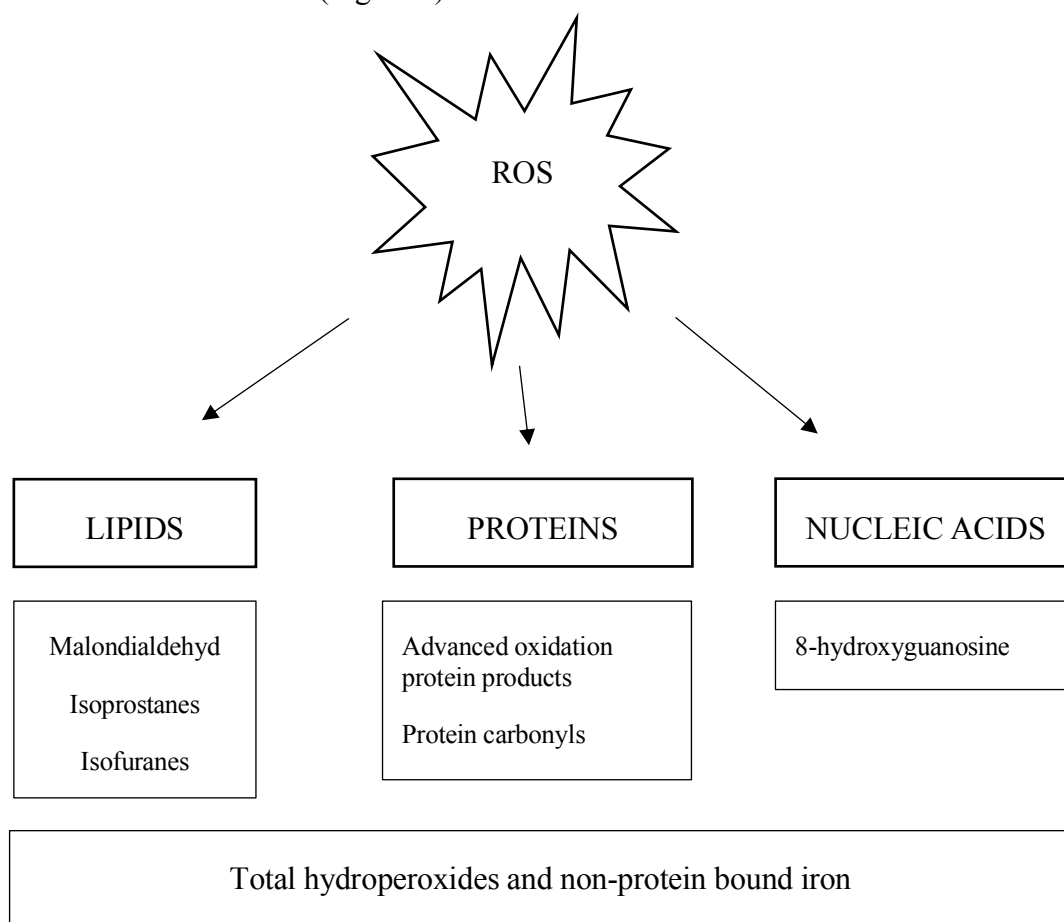


Figure 5. Known biomarkers of free radical damaged lipids, proteins and DNA respectively ⁷⁶.

The ratio of GSA/GSSG, to measure the amount glutathione that are oxidized is a technical challenge, and the biological processes work so fast that it will be difficult to sometimes interpret the result. Even so, it is one very central marker of oxidative stress and have been used in many studies. High levels of lipid hydroperoxide and low levels of GSH was correlated to mechanical ventilation and found in bronchoalveolar lavage fluid in preterm infants with BPD in comparisons to preterm infants without BPD ⁹⁰.

1.3.2 N-Acetylcystein amide (NACA)

N-acetyl cysteine (NAC) is a well-known and clinically widely used thiol antioxidant that functions as a potent scavenger of free radicals and facilitates the production of the intracellular antioxidant glutathione by reducing extracellular cystin to cysteine. When the carboxyl group of NAC is replaced with an amide group N-acetylcystein amide (NACA) is formed, a molecule that is neutral in charge with more lipophilic properties and thereby have better penetration through membranes including the mitochondria and the blood-brain-barrier. NACA restores cellular glutathione, have a potent metal chelating activity and prevent oxidative stress ^{91,92}, resulting in a stronger biological effect than NAC ^{93,94}.

There are different suggested mode of action for the thiol compound N-Acetylcystein amide (NACA). In a mouse model of asthma NACA attenuated airway inflammation using the NFκB pathway to inhibit VEGF and Th2 cytokine production and reduced hyper-responsiveness ⁹⁵. NACA could prevent cell death by blocking the p38 MAPK/iNOS signaling pathway *in vitro* and that was confirmed *in vivo* in a rat model of contrast-induced nephropathy ^{96,97}.

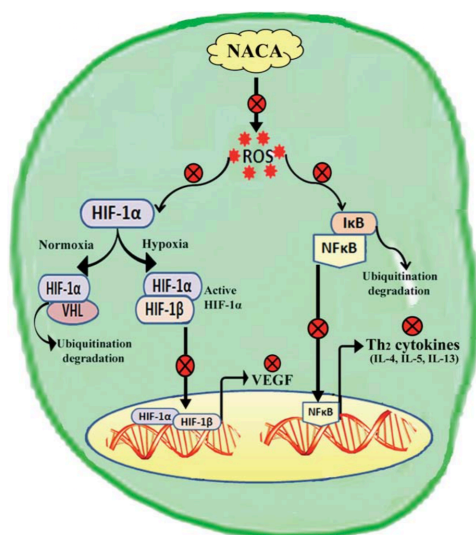


Figure 6. The suggested anti-inflammatory role of N-Acetylcystein amide (NACA) by inhibiting NFκB and HIF-1α which leads to suppression of VEGF and Th2 cytokines.

● The red circles represent possible mode of action of NACA.

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1.4 AGING

1.4.1 Telomeres

Telomeres are non-coding DNA sequences (TTAGGG) surrounded by telomere binding proteins, that stabilize the end of the chromosomes. The telomeres shortens with each cell division due to the end-replication problem ⁹⁸ and telomere length may therefore reflect cell

division history. Telomere length shortens faster when exposed to oxidative stress^{99,100} and is a marker of accelerated cellular aging^{101,102}. Telomere attrition rate could be slowed down *in vitro* by antioxidant treatment¹⁰³.

There is a great variability of telomere length between individuals but also within the same individual. At the same time, the telomere length correlate between different tissues within an individual. Therefore, telomere length in hematopoietic cells can serve as a surrogate marker for telomere length in other tissues¹⁰⁴.

SIRT1 is a nicotinamide adenine dinucleotide-dependent deacetylase that regulate different proteins involved in processes like inflammation, angiogenesis, production of ROS and senescence. Preterm birth was associated with low levels of SIRT1 and showed increased senescence¹⁰⁵. Critically short telomeres may induce cellular senescence and thereby reduce the risk for unreparable DNA damage. The tissue functions are influenced by the frequency of senescent cells. The pathway of signaling is not clear but stabilization of p53 involved in mitochondrial function and increased expression of p21 and SIRT1 has been suggested. Telomere dysfunction affect transcription factors that will activate p53 and result in mitochondrial dysfunction (metabolism, biogenesis, defective ATP generation and increased ROS production). If the mitochondrial respiration were mildly inhibited the pathways of longevity are activated, while pronounced impairment would trigger functional decline and aging¹⁰⁶.

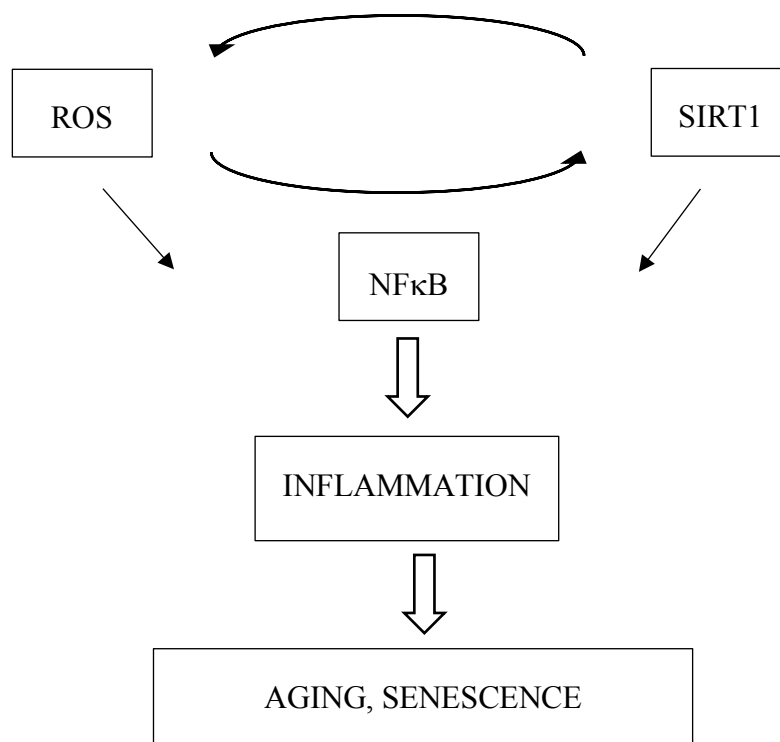


Figure 7. Pronounced impairment of mitochondrial function will promote inflammation and senescence. SIRT1 function as a metabolic sensor and can link the energy status of the cell to regulate gene expression and epigenetic changes.

Short telomeres have been linked to age related diseases in adults ¹⁰⁷⁻¹¹⁰. Studies of telomere length and prematurity in relation to disease are scarce but there is evidence of short telomeres associated to psychological stress in children ¹¹¹⁻¹¹³ or in metabolic conditions ¹¹⁴. Similar telomere length was found in a study of telomere length and lung functions ¹¹⁵ in a preterm cohort compared to term born children. Preterm born infants have longer telomeres at birth compared to term ¹¹⁶⁻¹¹⁸. There is still no answer whether preterm born individuals exposed to high levels of oxidative stress will age faster.

At birth humans have a telomere length of around 10 000 base pairs (bp) in their blood cells, around 3000 at 35 years of age and 1500 bp at 65 years of age. The entire chromosome has 150 million base pairs. On average a cell divides normally 50-70 times during its life time and at each division loses 30-100 bp ¹¹⁹⁻¹²¹.

Telomere length features an oscillating dynamic pattern ¹²² which levels out over time (Figure 8), but the underlying mechanisms have still not yet been unraveled ¹²³. Females tend to have longer telomeres than males.

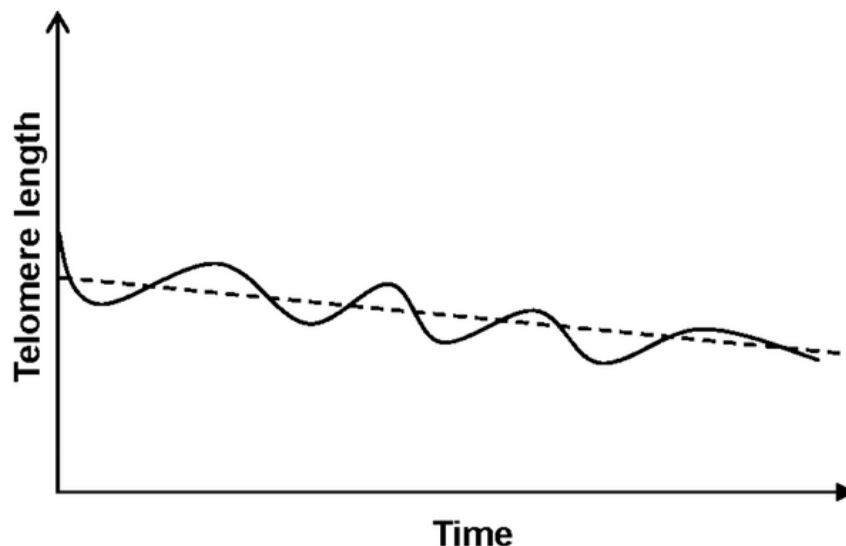


Figure 8. Svenson et al proposed the hypothesis of dynamic changes in telomere length over time ¹²².

The attrition of telomere length will differ in tissues but also during the course of life. The attrition rates vary in different age groups. Adults shorten their blood cell telomere length with ~30 bp/year, while children have been described to reduce theirs with 300 to 1000 bp/year ^{124,125}. The attrition rates in children seem to plateau around four years of age ¹²⁶. In preterm newborn infants the rate can be up to 270 bp/week ^{127,128}.

1.4.2 Telomerase

Telomerase is a ribonucleoprotein reverse transcriptase enzyme that maintains the telomere length by adding telomeric repeats to the end of chromosomes ¹²⁹. The telomerase complex contains several subunits including the telomerase reverse transcriptase (hTERT), its RNA component (hTERC) and associated proteins including dyskerin. Telomerase activity is high during fetal life, but decreases rapidly after birth except for in stem cells and in germline

cells. Telomerase plays a role in promoting cell growth and cell survival, and has shown to be active in signaling and mitochondrial function¹⁰⁶ and protect cells from oxidative stress^{130,131}. Telomerase activity was shown to be dynamic when adults were exposed to acute stress that gave a rise to increased cortisol levels, but also a significant increase in telomerase activity within one hour of the stress-exposure¹³². These findings imply that the decline of telomere length in hematopoietic cells can be reversed under specific circumstances and in response to specific stimuli.

1.4.3 DNA methylation

DNA consists of the different nucleotides thymine, guanine, cytosine and adenine. Our genes can be switched on or off by different mechanisms whereby epigenetic regulation is one. Epigenetic modifications are dynamic and may, without altering the DNA sequence, silence or activate genes. DNA methylation is one epigenetic mechanism that involves the addition of a methyl group (CH₃) to a carbon atom on a cytosine followed by a guanine (called CpG sites). The reaction is catalyzed by DNA methyltransferases. CpG rich areas of the genome are called CpG islands and are particularly sensitive for gene silencing if methylated. DNA methylation is an important regulator of cell functions and development but is also important for maintaining chromosome stability. Dysregulated DNA methylation has been associated with diseases, for instance in cancer. Much effort is put in trying to understand the role of hypermethylated and/or hypomethylated genomic regions in disease development and progression. Altered DNA methylation patterns can also be used as biomarkers for different conditions.

2 AIMS

Overall aim

To study inflammation and oxidative stress in preterm children and to better understand the mechanisms that triggers these phenomena aiming for future development of protective treatments to prevent inflammatory complications of preterm birth such as acute and chronic lung disease.

Specific hypothesis and aims

Study I

Preterm children have longer telomeres at birth compared to term born children. We hypothesized that children born preterm with a history of BPD would have shorter telomere length, as they were exposed to neonatal inflammation and at risk of growing up with impaired lung function. The aim was to evaluate the consequences of preterm birth and bronchopulmonary dysplasia at ten years of age in regards to inflammation, lung function and telomere length.

Study II

Prematurity itself and neonatal intensive care will trigger inflammatory processes and oxidative stress. Telomere length is a marker of oxidative stress. We hypothesized that preterm birth results in faster telomere attrition rate. The aim was to study if cellular aging, measured as telomere attrition rate and predicted DNA methylation biological age, was accelerated during the first two years of life following preterm birth and if early respiratory viral infections contributed.

Study III

Preterm born children are susceptible to infections during the neonatal period in life. We hypothesized that the immune system is hampered by preterm birth due to a different cell composition affected by immaturity. The aim was to study the immune system during the first three months of life to describe the immune development in preterm and term infants during their immunological transition to extrauterine life.

Study IV

Mechanical ventilation and inflammation inactivates pulmonary surfactant. We hypothesized that hyperoxia-induced surfactant inactivation can be prevented and lung function improved, using exogenous surfactant as a vehicle for treatments with an antioxidant. The aim was to study the consequences of hyperoxia on pulmonary surfactant in a preterm rabbit model and if inactivation of surfactant could be prevented by adding an antioxidant (N-Acetylcysteine amide) to the exogenously instilled surfactant treatment.

3 MATERIALS AND METHODS

3.1 SUBJECTS AND STUDY DESIGN (PAPER I, II AND III)

This thesis involves three clinical studies (I-III) with three different study cohorts and each cohort was part of a larger study. Study I was a cross-sectional cohort study of ten-year old children while the other two studies were longitudinal cohort studies from birth and up to around 4 months of age (study III) or up to two years of age (study II). In all clinical studies the outcome is put in relation to prematurity.

3.1.1 Paper I - Telomere length, lung function and inflammation

This cross-sectional cohort study was part of a larger follow-up study of preterm born children with BPD, the Premature follow-up with Lung function Mannitol and Methacholine (PULMM) study ³⁷. Children born in Stockholm County between 1998 and 1999 diagnosed with BPD were invited to a follow-up examination at 10 years of age. BPD was defined as the need for oxygen for 28 days postpartum and was graded into mild, moderate or severe at 36 weeks corrected gestational age, according to the definition by Jobe and Bancalari ⁸. Mild BPD was defined as no breathing support, moderate BPD as the need for supplementary oxygen of < 30% and severe BPD as the need for supplementary oxygen of 30% or more and/or ventilatory support. Outpatients at the Pediatric Asthma and Allergy department at Karolinska University Hospital were recruited as controls and consisted of gender- and age-matched children with allergic asthma born at term and without respiratory symptoms in the neonatal period. The follow-up investigations included blood sampling and lung function testing and all individuals with available blood sample were included in our study of which 29 were children born preterm with a history of BPD and 28 children as controls born at term with phadiatop positive asthma developed during childhood (Figure 9). Perinatal information was retrieved from the Medical Birth Registry and neonatal morbidity parameters were extracted from hospital records. We analyzed relative telomere length in relation to prematurity, lung function and inflammation.

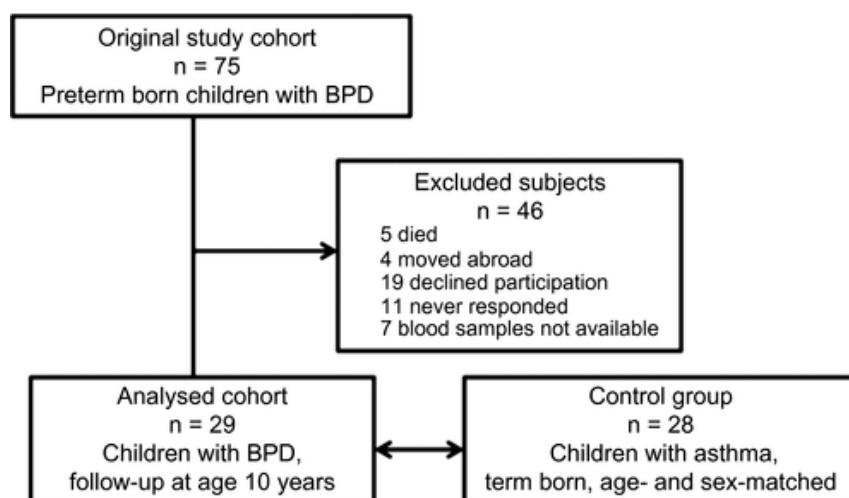


Figure 9. Flow chart of cohort ¹.

3.1.2 Paper II - Cellular aging and viral respiratory infections

A subgroup of preterm born children ($n=16$) and term born controls ($n=44$) with longitudinal blood samples were identified from a larger longitudinal case-control study of lung function development during the first two years of life, the LUFT study. LUFT was a follow-up study of lung function in preterm and term born infants and the impact of viral infections during the first year of life. Eligible for inclusion in the LUFT study were infants born preterm (before gestational week 36⁺⁶) during January 2009 to March 2011 and admitted to one unit at Karolinska University Hospital. Exclusion criteria were children with major malformations or neurological impairment preventing later lung function testing. The control group were healthy children born at term (gestational week 37⁺⁰ – 41⁺⁶) on the same day as the index-child and matched for gender and maternal smoking. The protocol included blood sampling at two time points; cord blood at birth and peripheral venous blood at follow-up at two years of age, as well as lung function evaluation at three months of age and at two years, but data from lung function is not reported in this thesis. Respiratory infections during the first year were monitored and in case of an infection also sampled. From the 197 children included in the LUFT study with at least one blood sample taking, we included 60 children with longitudinal samples for analyses of cellular aging in relation to prematurity and viral infections during the first year of life (Figure 10).

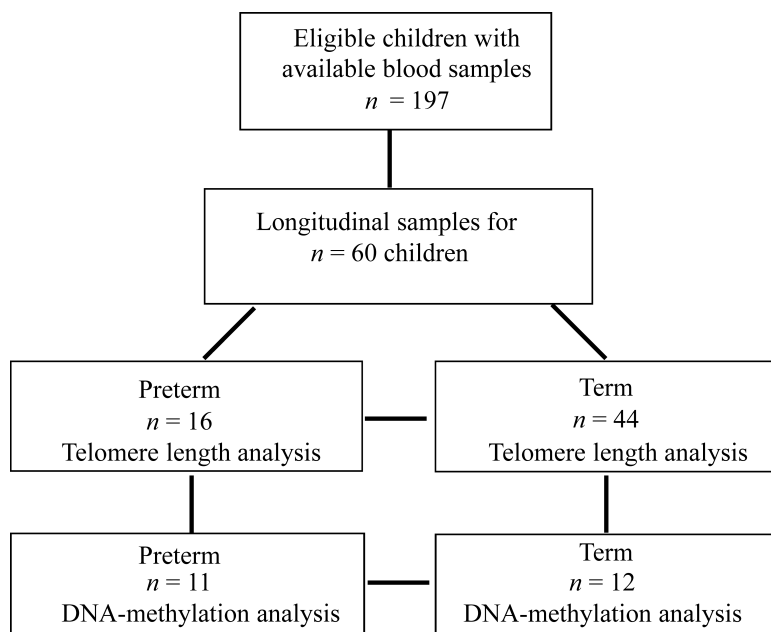


Figure 10. The study cohort of Study II. The analyzed subgroups children from a larger cohort study of lung function development during the first two years of life including preterm born and matched term born children at Karolinska University Hospital from 2009 to 2011. Children with longitudinal blood samples available from both birth and at 2 years of age were included in the telomere and cellular aging analyses (Paper II).

Cellular aging was measured with two different methods; as telomere attrition rate ($n=60$) and epigenetic aging (DNA methylation age, $n=23$). For selection of the smaller group to analyze predicted DNA methylation biological age (DNAm) we initially included matched pairs (10 preterm and term individuals). Due to difficulties in getting longitudinal samples and enough amount of extracted DNA, we included an additional 13 individuals, chosen by

the investigators to balance the groups for gender, parity, quarter of birth, mode of delivery and chronological age at follow-up. Perinatal information and neonatal morbidity data were retrieved from medical records.

3.1.3 Paper III - Neonatal immune system development

A subgroup (n=100 infants, 110 parents) from the study TELLUS (TELOmeres, LUng disease and oxidative Stress in preterm born infants) were included (Figure 11). Inclusion criteria in TELLUS are children born preterm before gestational age week 30 and gender matched term born controls (37⁺⁰ – 41⁺⁶) at Karolinska University Hospital from April 2014 and inclusion is still ongoing. Cord blood and placenta biopsy samples are collected at birth and blood, buccal cells, urine and faeces during the first four months of life (week 1, 4 and at term around week 12). Parental samples from blood and buccal cells are taken for hereditary comparisons and breast milk from mothers at every time point her child was sampled. Perinatal data was retrieved from medical records.

TELLUS (TELOmere LUng disease and oxidative Stress in preterm born infants)

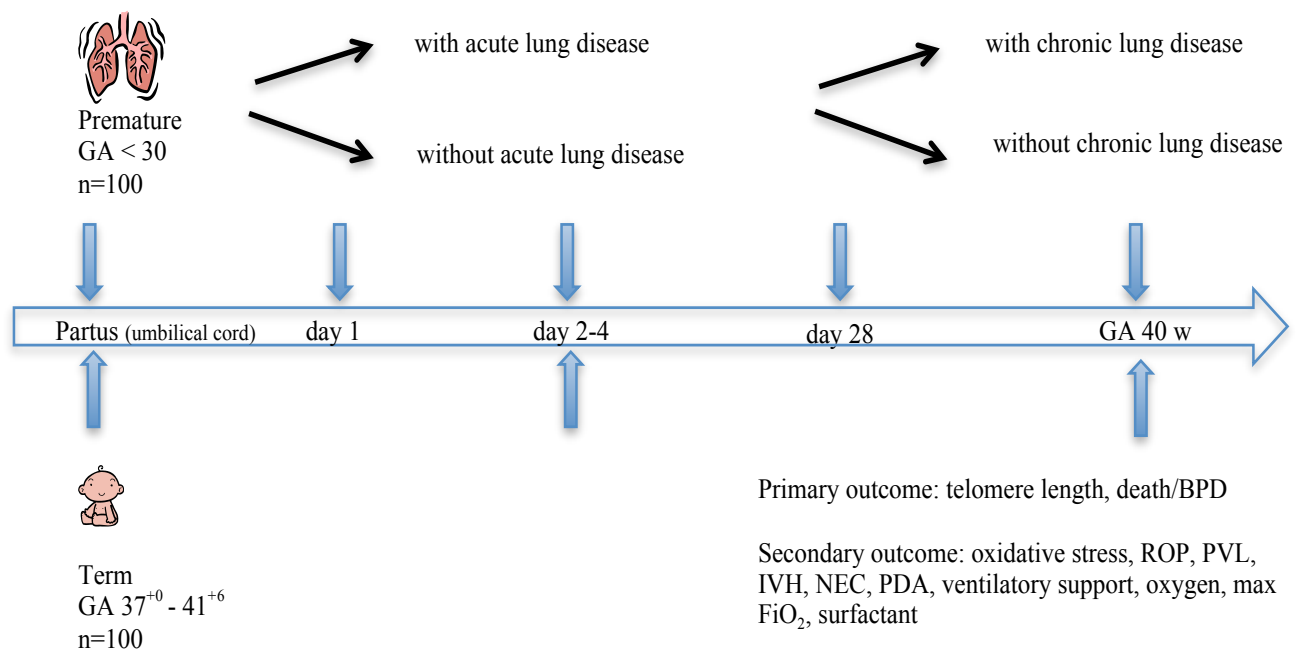


Figure 11. Study protocol of the on-going TELLUS study of which 100 infants (50 preterm and 50 term) was included in studies of neonatal immune system development¹³³. Cellular (biological) aging and oxidative stress will not be analyzed until the study is closed.

For Study III we enrolled 100 newborn children born between April 2014 and July 2017 and used blood and fecal samples. In all we used 285 samples from 100 children, 156 samples from 58 mothers and 52 fathers from the TELLUS cohort. In addition, 12 samples from 3 healthy adult controls were included.

3.2 TESTS AND MEASUREMENTS (PAPER I, II AND III)

3.2.1 Lung function (paper I)

Dynamic spirometry performed with a Vitalograph 2120 (Ennis of Ireland, County Clare, Ireland) was performed to assess pulmonary function before bronchodilation in study I. Forced vital capacity (FVC), forced expiratory volume in one second (FEV₁) and forced expiratory flow (FEF) using European Respiratory Society and Polgar reference values were determined¹³⁴. A positive reversibility test was defined as an increase of at least 10% of FEV₁ after 0.5 mg inhaled terbutaline. Plethysmography (CareFusion, Bavaria, Germany) was used to measure lung volumes: vital capacity, total lung capacity, fractionated residual volume and residual lung volumes. Diffusion capacity for carbon monoxide was evaluated using a single breath technique. Reference values of Hedenström Solymar were applied for static spirometry values and diffusion capacity¹³⁵. Lung function values were reported as percentage of predicted value.

3.2.2 Cellular aging (paper I and II)

3.2.2.1 DNA extraction

Whole blood of 2 mL was collected in EDTA tubes and frozen at -80°C within one hour. Genomic DNA was extracted using the MagNA Pure LC instrument (Roche Diagnostics Scandinavia AB, Stockholm, Sweden). DNA yield and purity were determined spectrophotometrically (Thermo Fischer Scientific Inc., Wilmington, DE, USA).

3.2.2.2 Telomere length

Relative telomere length (RTL) was determined by the quantitative polymerase chain reaction method described by Cawthon et al.¹³⁶ with minor modifications¹³⁷. Briefly, each DNA was analyzed in triplicate wells in separate Telomere (TEL) and single copy gene (hemoglobin subunit beta, HBB, Gene ID:3043) reactions at two separate times (ABI7900HT instrument, Applied Biosystems). The TEL/HBB and telomere/single copy gene T/S values were calculated by the $2^{-\Delta C_t}$ method, where $\Delta C_t = C_{t_{TEL}} - C_{t_{HBB}}$. C_t refers to the threshold cycle. The RTL value for each sample was generated by dividing the sample T/S value with the T/S value of a T-lymphocyte cell line called CCRF-CEM, which was used as a reference and included in all runs. The inter-assay coefficient of variation was between 4 and 8%.

3.2.2.3 Relative telomere length (RTL)

In study I RTL was evaluated cross sectional at 10 years of age in two cohorts of children with lung disease, preterm born children with BPD and term born children with allergic asthma. In study II we measured RTL at birth and at two years of age in preterm born infants and term born controls. The method using relative telomere length is adjusted to a reference DNA, but RTL-values from the different studies in this thesis may not be comparable due to different batches of PCR reagents.

3.2.2.4 *Telomere attrition*

In study II we measured RTL at two time points, at birth and at follow-up at two years of age, and therefore telomere attrition during the first two years in life could be evaluated. Telomere attrition rate per year was as follows; $(RTL \text{ at follow up} - RTL \text{ at partus}) / (\text{chronological age at follow up in years})$.

3.2.2.5 *DNA methylation*

High density arrays covering 485,577 CpG sites (HumMeth450K, Illumina, San Diego, USA) were used for genome-wide methylation analysis. The included CpG sites are located in different genomic regions, but focused on promoter-associated regions and CpG islands. Briefly, 500 ng DNA was bisulfite converted by the EZ methylation gold kit (Zymo Research, Irvine, USA). To each array, 200 ng of bisulfite-converted DNA was applied, and the arrays were operated according to the manufacturer's instruction and scanned with the HiScan array reader (Illumina). The fluorescence intensities were extracted using the Methylation module (1.9.0) in the Genome Studio software (V2011.1). The quality of each individual array was evaluated with built-in controls.

3.2.2.6 *Epigenetic aging*

The epigenetic DNA methylation (DNAm) age was calculated based on 353 CpG sites, by the "epigenetic clock" prediction model described by Horvath^{138,139}. Delta age was determined by subtracting the individual's chronological age from the estimated epigenetic DNAm age at birth and at two years of age. Epigenetic aging (DNAm aging rate/year) corresponds to $(DNAm \text{ age at follow up} - DNAm \text{ age at baseline}) / \text{chronological age at follow up}$. Baseline in our study was birth and therefore represents chronological age 0 years.

3.2.3 Immune system (paper I, II and III)

3.2.3.1 *Inflammation (paper I)*

In study I we evaluated inflammation using exhaled nitric oxide (FeNO) reported as part per billion (Niox equipment, Aerocrine AB, Stockholm, Sweden)¹⁴⁰. Blood cytokines, IL-6, IL-8, TNF- α and IL-1 β , were analyzed by electro-chemiluminescence immunoassay routinely used by the Karolinska University Hospital Laboratory.

3.2.3.2 *Viral infections (paper II)*

In study II we evaluated exposure to viral infections during the first year of life. Respiratory infections were monitored. Parents kept a diary and were instructed to report to the hospital for clinical evaluation when their child showed symptoms of infection. Nasopharyngeal sampling for viral infections was performed at every infection episode and analyzed by routine PCR methods at Karolinska University Hospital Laboratory. The panel covered adenovirus, influenza A and B, parainfluenza 1, 2 and 3, respiratory syncytial virus, metapneumovirus, coronavirus (Oc 43, 229 E, NL 63, HKU1), enterovirus, rhinovirus,

mycoplasma pneumoniae and chlamydia pneumoniae. Episodes of wheeze, bronchiolitis and hospital admissions were reported.

3.2.3.3 System-level immune analyses (paper III)

Samples were taken at birth (umbilical cord blood) and then longitudinally at week 1, 4 and 12. Samples used in study III was collected from blood and faeces. Blood ~100 μ L was put in fixation solution (Cytodelics AB) at once and put in the -80°C freezer after 5-10 min. 500 μ L of blood was put in an EDTA vial. The samples were centrifugated within one hour and plasma was extracted and put in the -80°C freezer. PBMC were extracted using a protocol based on Ficoll density gradient separation within two hours and prepared with freezing media and put in the -80°C by slow freezing for optimal cell viability. Both parents were also sampled accordingly at week 1 and for mothers also at week 12 after delivery.

3.2.3.4 Mass cytometry

System-level analyses is the application of many simultaneous measurements to describe a complex process involving many immune cell populations and proteins ¹⁴¹. For such analyses of individual immune cells Mass cytometry is a powerful method ¹⁴². This method is based on the use of about 40 different antibodies targeting intracellular and surface proteins in millions of individual immune cells to describe their overall phenotypes and function (Figure 12). We used this method to understand the neonatal immune system development in detail. We designed a mass cytometry panel with 38 antibodies targeting activation and differentiation markers across all white blood cell populations and profiled a total of 95,278,466 immune cells from 337 blood samples in total with as little as 100 μ L of blood.

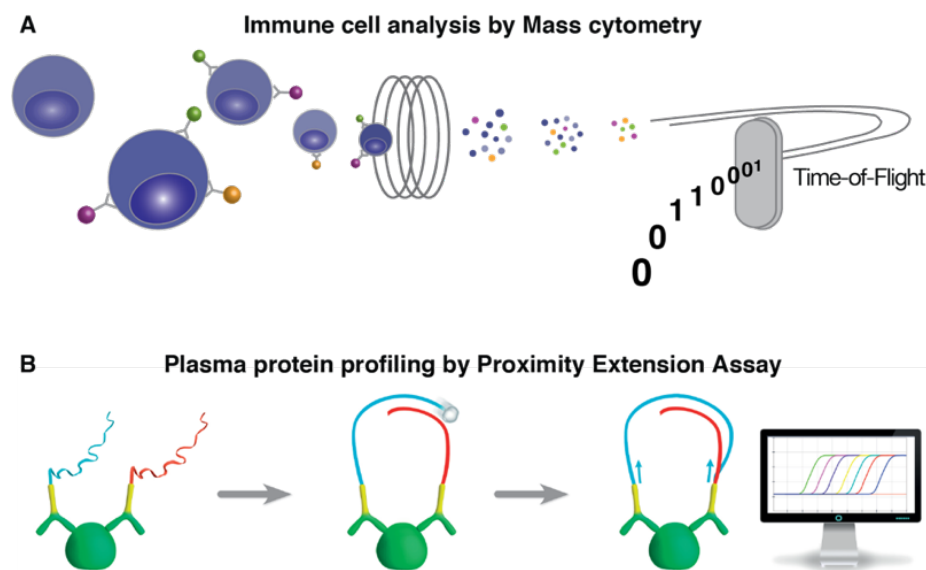


Figure 12 **A)** High-dimensional cell profiling by Mass cytometry in which 38 antibodies are used to target proteins in millions of cells. Each antibody is coupled to a unique-mass tag that can be quantified in an IPC-MS type system. **B)** High-dimensional plasma profiling by dual-recognition and qPCR readout after DNA-ligation of complementary oligos.

3.2.3.5 *Plasma proteins*

We used a sensitive dual-recognition immunoassay^{143,144} (ProSeek, Olink, Uppsala, Sweden) allowing for quantification of 267 unique proteins in < 20 µL of plasma.

3.2.3.6 *Transcriptome*

We used transcriptome analyses by PBMC mRNA-sequencing at weeks 1 and 12 to interrogate gene expression changes occurring after birth.

3.2.3.7 *Microbiome*

We performed 16S rRNA profiling of fecal samples from 45 children collected at weeks 1, 4 and 12 of life (n=95).

3.2.3.8 *Comprehend the big data*

The millions of data-points collected over time in the newborn children requires novel informatics pipelines for storage, processing and analysis. We built a relational database to manage all the acquired data and one relational database capturing clinical metadata. Analyses of cell populations by Mass cytometry involve quality control, filtering, gating on DNA-containing events (cells), removal of debris and classification of individual cells into known immune cell populations. As for plasma protein analyses, similar quality control steps were required and batch correction prior to integration of data types. The main method used to integrate cell and protein data is Topological data analysis¹⁴⁵ in which samples (both proteins and cells) are compared using correlation as a notion of similarity and placed in a parameter landscape that recreates early life immune system development. The remaining methods of analyses are described in detail in the publication and its supplementary method. Also, all scripts used as well as raw data are available (<https://brodinlab.com/newborns/>)

3.3 THE EXPERIMENTAL RABBIT MODEL AND STUDY DESIGN (PAPER IV)

Study IV is an experimental study using a preterm rabbit model of respiratory distress syndrome (RDS) conducted at the Surfactant Research Laboratory, Karolinska Institutet, Stockholm, Sweden.

3.3.1 **Surfactant preparations**

The surfactant used was porcine surfactant (poractant alfa, trade name Curosurf®) 80 mg/mL from Chiesi Farmaceutici, Parma, Italy. N-acetylcysteine amide (NACA) was provided by Dr. Glenn Goldstein (David Pharmaceuticals, New York, NY, USA). In the experiment the antioxidant NACA was dissolved with sodium chloride to a concentration of 100 mg NACA/mL and added to surfactant 80 mg/mL giving a NACA concentration of 0.4, 1.2 and 4 mg/mL. The concentrations of NACA in the surfactant suspension are 2.5, 7.5 and 25 mmol/L (mM) and surfactant of around 75 mg/mL. The NACA-surfactant suspension was prepared fresh before every experiment in room temperature.

3.3.2 Animal experiments

We used a well-established model to test surfactant in mechanically ventilated preterm surfactant deficient *New Zealand White* rabbits ¹⁴⁶ with some modifications of the protocol. The experiments were performed on 15 litters with a total of 110 rabbit pups with exclusion criteria of birth weight less than 20 or more than 40 grams (n=4), being stillborn (n=1), technical problems (n=2) or complications with pneumothorax (n=15), leading to an over-all survival of 80%. The rabbit pups were obtained sequentially by hysterotomy at a gestational age of 27 days (term 31 days). The pups were anesthetized with an intraperitoneal injection of pentobarbital sodium (0.1 mL, 6 mg/mL) and locally applied Xylocain on the throat for surgical preparation. After tracheotomy muscle relaxation was obtained with pancuronium bromide (0.1 mL, 0.2 mg/mL) given as an intraperitoneal injection and thereafter exogenous surfactant in the dose of 200 mg/kg was given intratracheally. The animals were placed in plethysmograph boxes at 37 °C connected to a ventilator system (Servo Ventilator 900 B, Siemens-Elema, Solna, Sweden or Stephanie, Stephan, Gackebach, Germany) and ventilated at a frequency of 40/min and inspiration/expiration ratio 1:1 (Figure 13). During the experiments individual pressure curves, tidal volumes and electrocardiography were recorded using a Powerlab system (AD Instrumental Limited, Chalgrove, Oxfordshire, UK) including Powerlab 4/20 (ML840), Bridge AmpT (ML110), Animal Bio Amp (ML136) and Spirometer (ML140). The animals were sacrificed at the end of the experiment with an intracerebral injection of lidocaine (2%, 0.5 mL) leading to an instant heart arrest and death.

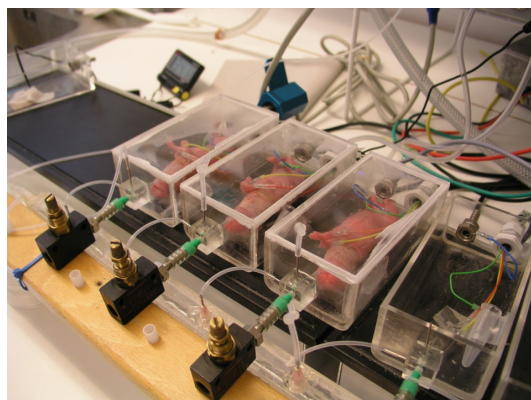


Figure 13. Newborn rabbits ventilated in parallel in plethysmograph boxes using individualized peak inspiratory pressures to achieve tidal volume of 6-8 mL/kg.

Experiment 1: Preterm rabbits (n=27) were treated at birth with 0.5, 1.5 and 5% NACA in surfactant. Animals receiving the same dose of surfactant served as positive controls and non-treated littermates as negative controls. The surfactant dose was 200 mg/kg. The newborn rabbits were ventilated in parallel with standardized sequence of peak inspiratory pressures (PIP). To open up the lungs, pressure was first set at 35 cmH₂O for 1 min. After this recruitment maneuver, pressure was lowered to 25 cmH₂O for 15 min, 20 cmH₂O for 5 min and 15 cmH₂O for another 5 min. Finally, pressure was raised again to 25 cmH₂O for 5 min. The experiments were performed without positive end-expiratory pressure (PEEP).

Experiment 2: The animals were randomized to mechanical ventilation with either 21% (n=10) or 100% oxygen (n=11) and treated with porcine surfactant (200 mg/kg). The treated animals were ventilated for 90 min with a PEEP of 3-4 cmH₂O and individualized peak inspiratory pressure to achieve a tidal volume of 6-8 mL/kg. The untreated control animals (n=3) were only ventilated for 30 minutes. Some animals received surfactant and was not ventilated (n=6) to serve as a baseline for what ventilation can cause to oxidative stress markers.

Experiment 3: The animals were randomized to mechanical ventilation with 100% oxygen and treatment with surfactant (n=15) or 1.5% NACA in surfactant (n=13). The treated animals were ventilated for 60 min with a PEEP of 3-4 cmH₂O and individualized PIP to achieve a tidal volume of 6-8 ml/kg. The untreated controls were ventilated for 30 min (n=2) and one animal served as baseline.

3.4 TEST AND MEASUREMENTS (PAPER IV)

3.4.1 Lung compliance and lung gas volume

Dynamic lung compliance was calculated from pletysmographic pressure-volume registrations at 15, 30, 45, 60 and 90 minutes. Volume and pressure were recorded at start, 10, 20, 30, 45, 60, 75 and 90 minutes. Tidal volume (V_t) was divided with peak inspiratory pressure and body weight.

The lung gas volume (LGV) used in experiment 1 can also be described as the functional residual capacity and can be calculated by the difference between the organ volume of the lung and the tissue volume. By dipping the lung into water, the water will be displaced and the weight of that volume will represent the lung volume. Tissue volume was retrieved by converting the wet-weight of the lung to a volume using a constant (1.077 with standard deviation of 0.03). The formula to calculate LGV (mL/kg) = (lung volume (mL) – (wet weight (g)/1.077))/body weight (kg).

3.4.2 Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed after euthanasia by installation of 20 or 40 mL/kg of normal saline and retracting it three times. The procedure was repeated five times and the washes were pooled. Aliquots of the BAL fluid was ultra-centrifugated to separate the phospholipids as large (LA) and small aggregates (SA) and markers of oxidative damage were analyzed both in BAL before centrifugation and in BAL after ultra-centrifugation in the LA and SA portion separately.

3.4.3 Biophysical activity of surfactant

Biophysical activity of surfactant was tested in vitro by mimicking the alveolus of the lung in the pulsating bubble surfactometer (Surfactometer International, Toronto, Canada). A bubble of air in contact with the surrounding was inserted in a chamber filled with surfactant and pulsated using a piston system. Dynamic surface tension was recorded at 37°C during 50%

cyclic compression of the bubble surface and at a frequency of 40 cycles per minute. All measurements were performed for 5 minutes and at a lipid concentration of 5 mg/mL. The pressure at specific time intervals were measured and using the law of Laplace the surface tensions at minimum (γ_{\min}) and maximum (γ_{\max}) bubble size was calculated¹⁴⁷.

3.4.4 Hyperoxic damage of surfactant

3.4.4.1 Phospholipid determination

The phospholipids (phosphatidylcholine, lysophosphatidylcholine, sphingomyelin) were determined by an enzymatic method (LabAssay™ Phospholipid, Wako Pure Chemical Industries, Ltd, Osaka, Japan). Surfactant have different structural forms of which around 85% consists of more surface-active large aggregates (LA) that are involved in stabilizing the alveoli during breathing and degrades to smaller less surface-active aggregates (SA) normally during respiration. When the lung is injured the amount of SA increases leading to a disturbed SA/LA ratio and we used this to assess surfactant inactivation in the experimental model of respiratory distress syndrome and exposure to hyperoxia. Bronchoalveolar lavage fluid was centrifugated (40,000 x g for 60 min at 4°C)¹⁴⁸ and phospholipid content was determined in BAL both before centrifugation and then in the LA and SA components separately.

3.4.4.2 Lipid peroxidation

The degree of lipid peroxidation in the BAL fluid was determined by measuring the amounts of malondialdehyde (MDA) and 4-hydroxyalkenals (4HNE), using a LPO-586 colometric assay (Bioxytech LPO-586, OXISResearch, OXIS Health Products, Inc., Portland, USA)^{149,150}. The maturity can differ between litters in the animal experiments and enzymatic kits are known to be sensitive to react differently between batches. We compensate for this by calculating a score of lipid peroxidation (LPO score) using the amount of MDA per amount of phospholipids (mmol MDA/mg PC) for each animal divided by the mean value of the litter¹⁵¹.

3.4.4.3 Total proteins and protein carbonyls

Total protein content was quantified in the BAL fluid using the BCA Protein Assay kit (Immundiagnostik, Bensheim, Germany). Protein carbonyls are formed through oxidation of proteins and was quantified by an assay using ELISA (BCA-Protein Quantification Assay for the Carbonyl Protein ELISA, Immundiagnostik, Bensheim, Germany) measuring carbonyls in micrograms of proteins.

3.5 STATISTICAL ANALYSES

In study I, II and IV, data are given as mean and standard deviation when normally distributed, and as median and interquartile range otherwise. ANOVA and independent sample t-tests were used for normally distributed data. Levene's test for equality of variance was performed and, if significant, a t-test assuming different variance was performed. Non-

normally distributed data were analyzed using Kruskal-Wallis' rank-sum test. Differences were tested with chi-square and Fisher's exact test for categorical data and we used Pearson coefficients for correlation analysis. In paper I and II linear regression was used for evaluating group differences between preterm and term born children using perinatal data (for instance maternal age, premature rupture of membrane, parity, mode of delivery, birth weight, BMI and gender), evaluating inflammation and lung function (study I) and postnatal viral infections (study II). In study I linear regression was used to analyze relative telomere lengths in relation to neonatal morbidity in the preterm group using an F-test for categorical predictors with more than two categories.

In study II methylation data were pre-processed and normalized by BMIQ method as previously described ¹⁵² using R (v2.15.0). The methylation levels (i.e., the β value) of each CpG site ranges from 0, corresponding to completely unmethylated DNA, to 1, representing fully methylated DNA.

For study I, II and IV statistical analyses were also performed using STATA version 13 (StataCorp, College Station, TX, USA) and IBM SPSS Statistics version 25 (IBM corporation, Armonk, NY, USA). A p-value < 0.05 was considered statistically significant.

Study III involves a massive dataset and hundreds of different statistical tests. All these analyses were performed by a team of computational biologists within our collaborator Petter Brodin's research team using custom built pipelines mainly written in R and Python. All these methods are described in detail in paper III.

3.6 ETHICAL CONSIDERATIONS

For clinical studies (study I, II, III) written parental consent was obtained. Parents were approached after assent of the attending physician. In study III samples from cord blood and placenta was retrieved and if the parents decided not to participate in the study the samples were discharged. No samples were taken in any baby without the parents had given their written and oral consent. Studies in children always involve particular ethical considerations given that children themselves are never allowed to consent in instead represented by their parents. In the study all blood sampling was performed concomitant with clinical sampling whenever possible to minimize the number sampling procedures and the blood volume sampled was always kept to a minimal required to perform assay. The studies in this thesis was approved by the Regional Ethical Review Board in Stockholm for study I, II and III; and from the Animal Ethical Committee in Stockholm for study IV.

4 RESULTS

4.1 PAPER I

We related relative telomere length (RTL) to prematurity, lung function and inflammation. Telomere lengths were similar in 10-year-old children irrespective of a history of preterm birth, BPD or childhood asthma. However, there was a gender difference with females having longer telomeres than males at 10 years of age whether born preterm or term (Figure 14).

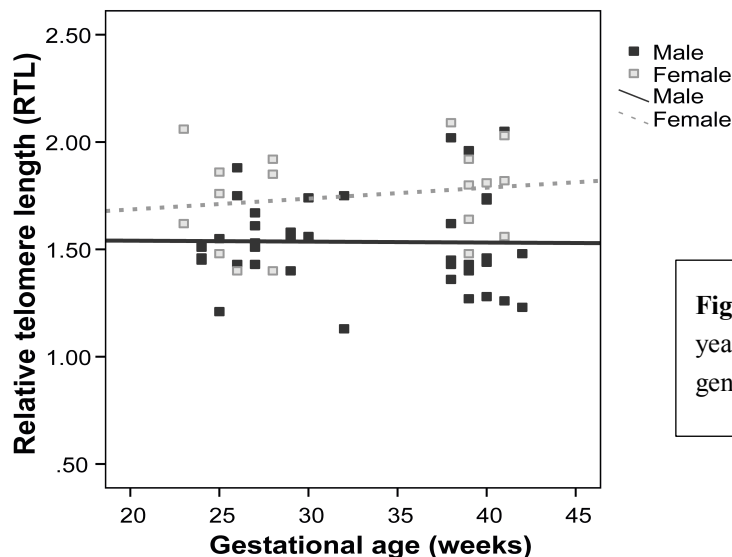


Figure 14. Relative telomere length (RTL) in 10-year-old children in relation to gestational age and gender ¹.

The term born group (n=28) that developed allergic asthma during childhood had a mean gestational age at birth of 40 weeks and a birth weight of 3591(range 2925 to 4210) grams. The preterm born group with a history of BPD (n=29) had a mean gestational age of 27⁺¹ (23⁺² – 32⁺⁵) and a mean birth weight of 1070 (607 to 2426) grams. Almost 60% had moderate or severe BPD, required a mean of 10 days with ventilatory support and 60% of the preterm infants needed CPAP more than 35 days (range 7 to 70 days). A majority received antenatal steroids (86%) and 62% were treated with surfactant. One third needing surgery for severe ROP and two thirds were treated for PDA but no infant had severe IVH (grade 3-4)¹⁵³. More than half were diagnosed with sepsis and a majority were treated with postnatal corticosteroids (86%).

RTL did not correlate to perinatal factors such as gestational age, birth weight, SGA, mode of delivery, parental age or parity. We found no correlations to physiological parameters at the age of 10 years such as age at follow-up, weight, length, BMI or blood pressure. In the preterm born group neonatal morbidity (ventilatory support requirements, surfactant administration, duration of supplemental oxygen, neonatal sepsis, IVH, ROP, PDA, severity of BPD) did not affect RTL.

Lung function on the other hand, did affect RTL. Our two groups both had an impaired lung function at 10 years of age compared to normal reference values ¹³⁴. Preterm children with BPD showed significantly lower results than term children with asthma for both dynamic and

static spirometry. It was most pronounced in the smaller airways with reduced FEF_{50%} ($p < 0.01$) and impaired diffusion capacity ($p < 0.05$).

RTL showed a positive association to dynamic spirometry indices (FEV₁/FVC, FEF_{50%} and FEF_{25%}) in the crude linear regression analysis but after adjusting for gender this association only remained significant for FEF_{50%} (regression coefficient = 0.003, $p = 0.03$), and if divided in groups, only for the asthma group alone (regression coefficient = 0.004, $p = 0.02$).

There was no difference when evaluating inflammation measuring blood cytokines (IL-1 β , IL-6, IL-8 or TNF- α) between groups. Airway inflammation measured as fractional exhaled nitric oxide was more prominent in the group of children with asthma (23.7 ± 21 versus 12.2 ± 9 , $p < 0.05$) compared to the BPD group, but we found no correlation between RTL and inflammation.

We concluded that blood telomere length at the age of 10 years was not shorter in children with a history of preterm birth and BPD compared with term born children with allergic asthma. Impaired lung function with low forced expiratory flows and male gender were independently associated with shorter telomere length.

4.2 PAPER II

In this longitudinal cohort study we included 16 infants born preterm with a mean gestational age of 31^{+6} and birth weight 1894 grams and 44 infants born term (40^{+0} weeks and birth weight 3517 grams). The groups were matched for gender (57% males) but cesarean section was more common in the preterm group (44% versus 18%). No infants were born small for gestational age (SGA). Ethnicity, maternal age and smoking did not differ between groups; noteworthy is that only two mothers in the cohort were smokers. Preterm infants were significantly older (chronological age) than controls at the follow-up time point, with a mean postnatal age of 25.6 months (range 22 to 32) compared to 22.6 months in term infants (range 18 to 28). The expected difference in weight and BMI z-scores between preterm and term born infants at birth was no longer seen at two years of age.

Neonatal morbidity was low in this rather mature preterm cohort. Only one child needed oxygen for more than 28 days of life, but none required supplementary oxygen or ventilatory support at 36 weeks corrected gestational age. CPAP was needed for a mean of four days (range 0 to 27) and none required mechanical ventilation. Only six infants needed supplementary oxygen for on average four days (range 0 to 28) and surfactant treatment was given to three infants (19%). No infant had IVH grade 3-4¹⁵³ or needed surgery for NEC, PDA or ROP.

Preterm infants had longer RTL compared to term infants both at birth ($p < 0.05$) and at two years of age ($p < 0.01$). No gender effect was seen on RTL either at birth or at two years of age. We did see that preterm born females had longer telomeres than term born females at birth ($p < 0.05$) and preterm born males had longer telomeres than term born males at two

years of age ($p < 0.01$). RTL did not correlate to perinatal factors such as maternal age, premature rupture of membranes, parity, mode of delivery, birth weight or gender.

The exposure of respiratory viral infections during the first year of life was similar for children in both groups (94 versus 86%), however infants born preterm required more often admission to hospital and had more bronchiolitis compared to the control group ($p < 0.01$). Relative telomere length was, to our surprise, longer in children with infections compared to children without ($p < 0.05$).

Telomere attrition rate per year (RTL at follow-up – RTL at birth/chronological age at follow-up) over the first two years of life were similar for both groups. However, boys in the control group had an accelerated telomere attrition per year compared to boys in the preterm group (0.01 ± 0.1 in preterm versus -0.12 ± 0.2 in term, $p < 0.01$). RTL did not correlate to any perinatal factors or to neonatal respiratory morbidity.

Preterm infants were generally younger in predicted DNAm biological age at birth and at two years of age, but only statistically significant when comparing delta age (-0.13 ± 0.8 versus 0.65 ± 0.9 years, $p < 0.03$). Epigenetic aging showed a tendency to be slower in the preterm group but did not reach any statistical significance ($p = 0.08$). There was no correlation between epigenetic aging and telomere attrition rate in preterm and term born infants during the first two years of life.

In summary, despite early exposure to risk factors for accelerated cellular aging, children born preterm exhibited preserved telomeres and did not show accelerated epigenetic DNAm aging, suggesting active repair mechanisms compensating for stress during the neonatal intensive care period.

4.3 PAPER III

We enrolled 100 newborn children of which 50 were born preterm (< 30 weeks gestational age at birth) with a birth weight ranging from 458 to 1623 grams and 50 were born at term ($37^{+0} - 41^{+6}$) with a birth weight from 2838 to 4655 grams. Half of the children were males and 51% were born with cesarean section. The mothers had a median age of 32 (range 20-43) and the fathers a median age of 35 (range 21-52). The healthy controls were all males and of age 26, 28 and 36. Preterm children were admitted to the neonatal intensive care unit for a median of 65 days, with frequent antibiotic exposures, an increased risk of infections and developed complications typical for extremely preterm born infants. Term infants stayed at the hospital for a few days without complications and did not receive any antibiotics.

The main results from the longitudinal system-level analysis of the neonatal immune system reflecting the adaptation of newborn infants to postnatal life involves drastic changes in relative frequencies of diverse immune cells after birth. Preterm and term children differ in both cell composition and in plasma proteins at birth (Figure 15), for instance preterm delivery was associated with strong inflammatory response with a high expression of

CXCL11 and IL-8¹⁵⁴. Cell composition also differed between preterm and term, including a lower proportion of neutrophils and a higher proportion of T-cells in preterm infants.

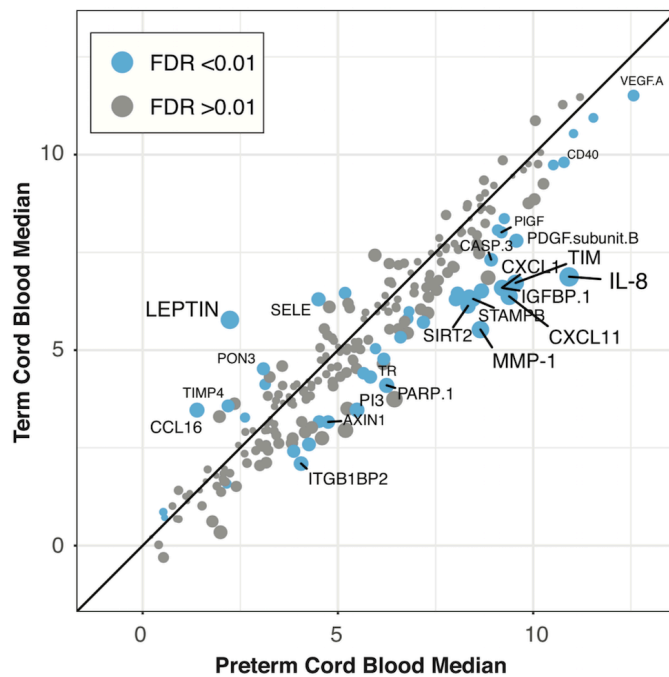


Figure 15. Protein concentrations in cord blood of term and preterm children. Differentially regulated proteins are marked in blue¹³³. (FDR=false discovery rate)

The cell composition, plasma proteins concentrations and cell phenotypes measured in cord blood was not representative of postnatal immunity. We compared the measurements from cord blood with samples taken during the first week of life (median, day 3) and calculated correlation coefficients to see whether cord blood could predict the pattern of cell frequencies seen at day 3. We found that only 6 out of 21 immune cells correlated strongly and also only one out of 81 plasma proteins with known immune function could be predicted from cord blood in the first week of life. This unpredictability could partly be explained by the different sources of samples (cord blood and peripheral blood), but the difference in immune cell composition during the first week of life was also seen in a few children (n=7) where we had peripheral blood both at day 0 and from week 1. This implicates that the inability to predict postnatal immune systems from cord blood was not just due to samples coming from different tissues, but can partly be explained by the drastic changes that occur in the cell composition right after birth.

Further, we were able to describe different phenotypic changes within each cell population. To be able to comprehend the many dimensions of single-cell data we used Barnes-Hut t-SNE (bhSNE) to create two-dimensional embeddings of single cell data from consecutive time points (Figure 16). Below we show that while blood samples taken from adults with one week in between show remarkable phenotypic stability, the immune cell phenotypes of neonates were markedly different when comparing cord blood with week 1 samples.

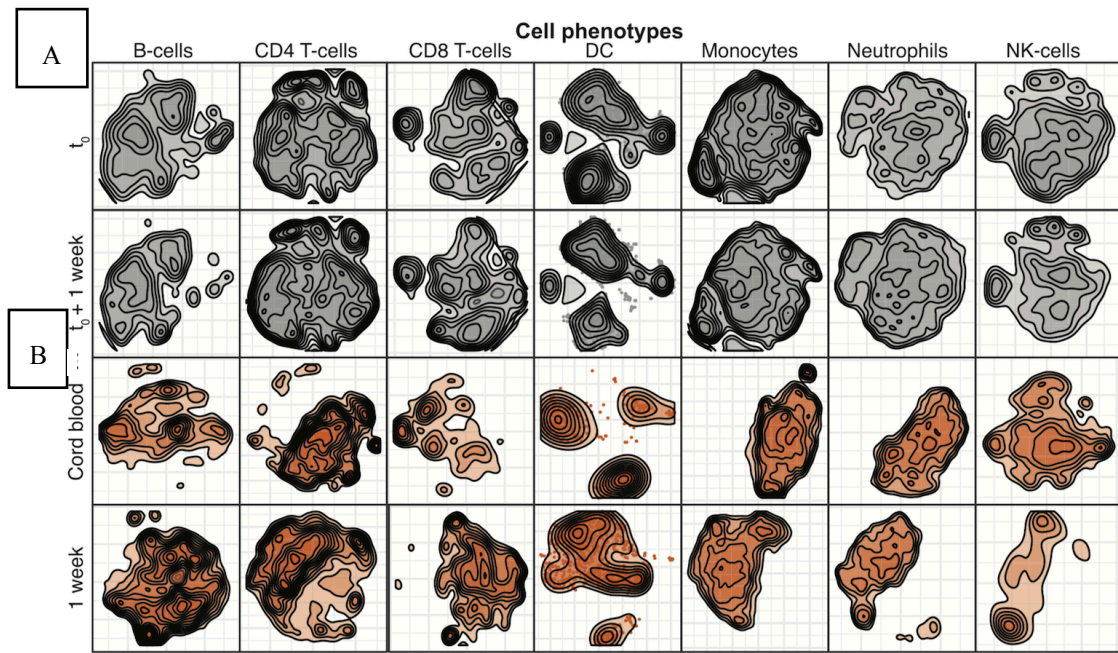


Figure 16 A) bhSNE embedding indicating cells in blood samples from two consecutive weeks in an adult. B) bhSNE maps of immune cell phenotypes in cord blood and week 1 from a newborn ¹³³.

Despite drastic changes in cell composition, plasma protein concentrations, cell phenotypes after birth, and differences between preterm and term born infants, the immune system rapidly converges following a stereotypic pattern. This was illustrated by integrating all data (cell frequencies, protein concentrations, clinical metadata) using topological data analysis to visualize the changes in several different measurement modalities over time (Figure 17). There were no large effects of gender or season of sampling on plasma protein concentrations or immune cell composition throughout the study period.

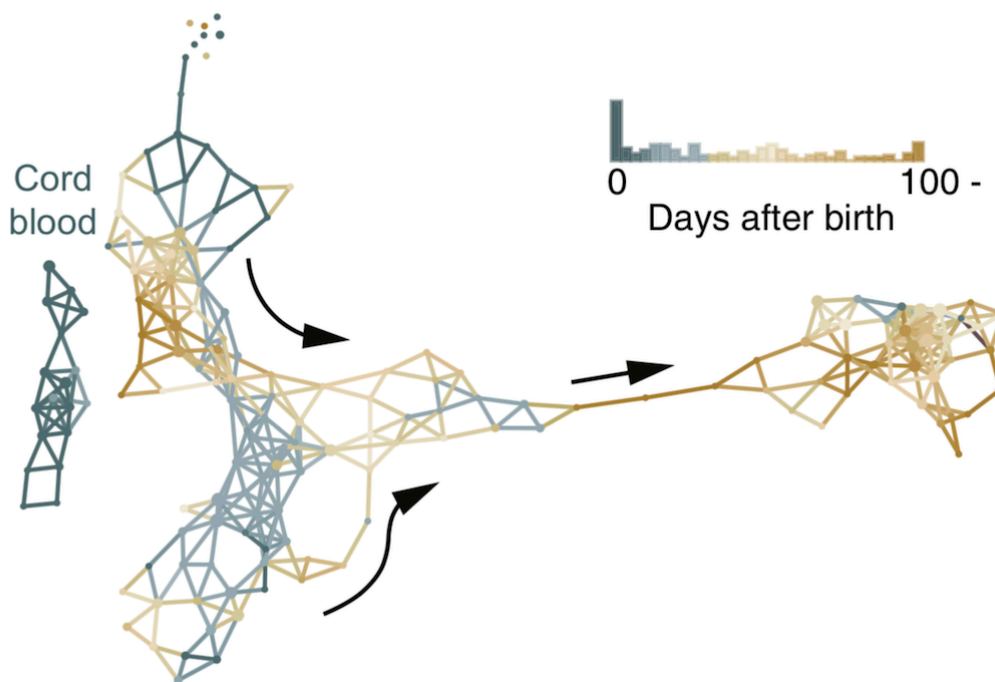


Figure 17. Topological landscape of neonatal immune system adaptation ¹³³.

The convergence of preterm and term immune systems was mostly explained by changes in the frequencies of neutrophils and naïve CD4+ T cells, which were seen already during the first weeks of life. However, we performed mRNA sequencing (mRNA-seq) of viable peripheral blood mononuclear cells (PBMCs) comparing preterm (n=4) and term (n=4) at week 1 and 12 at the transcriptome level. Also, with this analysis we found that the groups were largely intermixed, but that gene expression differences persisted at 12 weeks postnatally that could distinguish preterm from term. An analysis of gene ontology (GO) terms associated with those genes overexpressed in preterm infants found that those genes were involved in the negative regulation of interferon-gamma (IFN γ) production, T cell proliferation and IL-10 secretion. This is potentially important to explain differences in susceptibility to infectious diseases between preterm and term infants.

All cell populations vary over time, but the pattern for newborn children were very dynamic compared to adults (Figure 18).

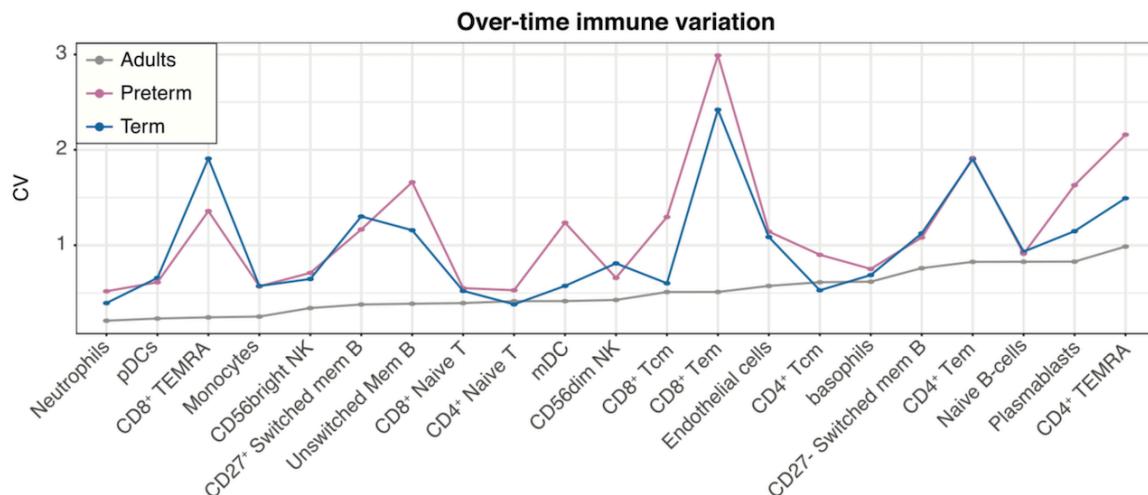


Figure 18. Variation of cell populations over time in adults and infants ¹³³.

We are trying to understand these changes over time which is demonstrated below (Figure 19) with specific examples of some cytokines (IL-27, IL-10, IL-8, IL-17A, IL-12B) and the transmembrane protein PlgR (polymeric immunoglobulin receptor). PlgR is important in immunoglobulin A transcytosis of epithelial cells and is thereby important in mucosal immunity. Patterns of the cytokines and the transcriptome data imply that the changes in the immune system involve metabolic pathways and important immune system processes related to the response to microbes exposed at the mucosal surface.

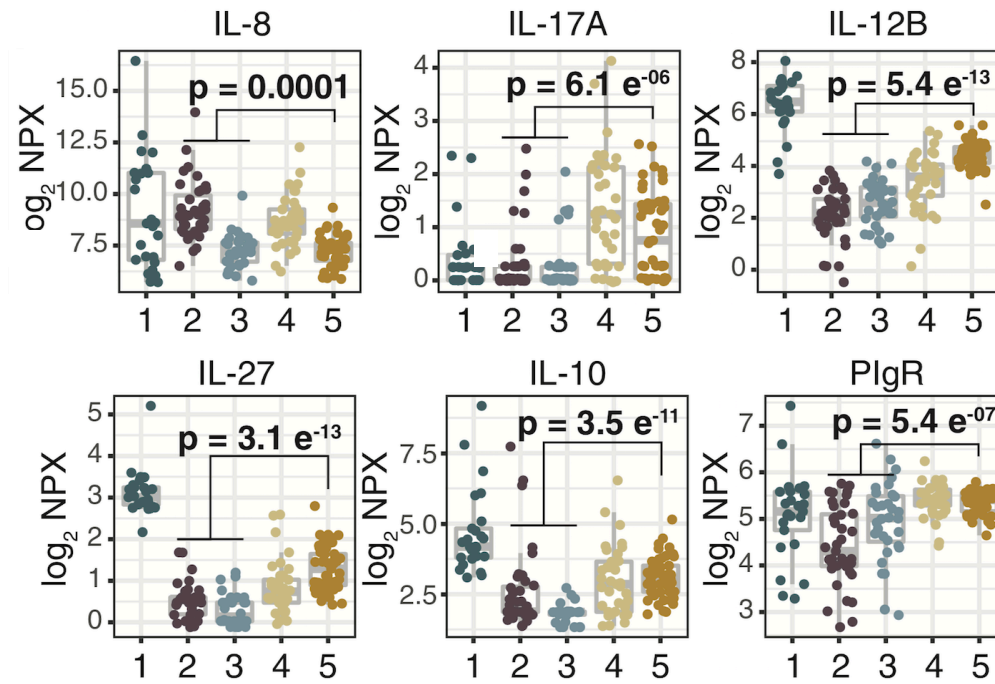


Figure 19. Plasma concentrations at different time points: 1=cord blood, 2=preterm early, 3=term early, 4=convergence, 5=late (3 months) ¹³³.

Finally, we performed 16S rRNA profiling of fecal samples from 45 infants at week 1, 4 and 12 (95 samples). Early dysbiosis showed a dominance of bacterial classes of Bacilli or Gammaproteobacteria, and more circulating endothelial cells, effector T cells populations and higher levels of the pancreatic exopeptidase CPA1 in their blood samples at 3 months of age. We suggest that microbial interactions early in life will affect the colonization of the child and thereby affect immune system development.

We conclude that cord blood is not representative of postnatal immunity and that preterm and term born infants differ at birth but rapidly converge to a shared trajectory of the immune system. This trajectory follows a stereotypic pattern possibly driven by microbial interactions and might be hampered by early gut dysbiosis.

4.4 PAPER IV

The biophysical activity of surfactant in the presence of N-acetylcysteine amide (NACA) was tested *in vitro* using the pulsating bubble surfactometer and *in vivo* (n=31) randomizing animals to different concentrations of NACA-surfactant concentrations evaluating dynamic lung compliance and lung gas volumes. Despite high concentrations of NACA given both *in vitro* or *in vivo*, there were no significant effect on the biophysical properties of surfactant including dynamic lung compliance and lung volumes.

We evaluated the effect of hyperoxia on oxidative stress measuring lipid peroxidation and structural changes of phospholipids evaluating the ratio between large and small aggregates (SA/LA ratio). In the normoxic group there were complications with overventilation leading to air leakage after 60 to 75 minutes of ventilation, which explain the better survival of hyperoxic animals (75% versus 64%). Despite this, hyperoxia affected the structure of

phospholipids showing increased production of small aggregates ($p < 0.05$) which imply less surface-active surfactant and there was a tendency of more lipid peroxidation (1.4 [0.02;0.08] versus 1.1 [1.0;2.0]) and impaired lung functions (0.42 [0.3;0.8] versus 0.70 [0.6;0.8]).

Finally, we wanted to evaluate if adding an antioxidant to the porcine surfactant could ameliorate the damage of hyperoxia in surfactant deficient animals. After the in vitro and in vivo experiments of NACA we used 7.5 mmol/mL of NACA in porcine surfactant 80 mg/mL. Due to the risk for overventilation seen in the normoxic animals we adjusted the ventilation time to 60 minutes. All animals were ventilated with 100% oxygen and were randomized to receive treatment with the NACA-surfactant suspension ($n=13$) or to surfactant alone ($n=15$). No difference in lipid peroxidation, protein degradation, ratio of small to large aggregates or in dynamic lung compliance could be detected.

In summary, treatment with NACA in the surfactant suspension did not ameliorate any consequences of hyperoxia that we could detect with our methods during this one-hour long experiment, but the surfactant function or treatment effect was not hampered and surfactant was not destabilized by adding NACA.

5 DISCUSSION

The main focus of this thesis is trying to understand the cellular mechanisms by which preterm birth influence the adaptation to extrauterine life and give rise to inflammatory complications. The four papers included involve different aspects of environmental exposures in children delivered preterm; telomere length, inflammation and lung function (paper I), viral respiratory infections and cellular aging using the biological markers telomere length (telomere attrition rate) and predicted DNA methylation biological age (paper II), immune system development and environmental exposures (paper III), and hyperoxia-induced lung damage in an experimental model and the capacity to counter-act surfactant inactivation with a novel antioxidant (paper IV).

The principle findings are that telomere length was similar in 10-year-old children born preterm with a history of BPD and term born children with allergic asthma. Impaired lung function with low forced expiratory capacity and male gender were associated with short telomeres irrespectively of preterm birth (paper I). Despite early exposures to risk factors, preterm born children had preserved telomeres and showed no accelerated epigenetic aging during the first 2 years of life (paper II). Measurements of immune system in cord blood was not representative of postnatal immunity. The immune system of preterm and term children differed at birth but unexpectedly converged early in life and followed a stereotypic pattern of adaptation to extrauterine environment. Microbial interactions of the environment may affect early immune system development (paper III). Hyperoxia impaired surfactant function in the experimental rabbit-model and this was not prevented by addition of an antioxidant N-Acetylcysteine amide. The antioxidant did not affect surfactant function or treatment effect. As a proof-of-concept, exogenous surfactant can serve as a vehicle for antioxidant treatment to the lung (paper IV).

The methodological considerations for this thesis mainly involve patient selection bias and the limited sample size. The selection bias is potentially influential since we used existing cohorts in paper I, II and III. If there are systematic differences in their characteristics that are not representative of the population in general, this selection bias might inadvertently influence the results. The small sample size was a consideration in all papers (I-IV).

In paper I, no healthy control group was included. Both preterm and term born children had an impaired lung function and this might elude the differences that potentially could be found in telomere length at 10 years of age. The term group diagnosed with asthma had more inflammation. We hypothesized that BPD was a neonatal exposure of inflammation and would result in shorter telomeres. With the cross-sectional study design the exposure of BPD as a neonatal inflammation and the exposure of inflammation due to asthma in childhood cannot be differentiated and might attenuate the potential differences in telomere length due to prematurity. Another problem was the predefined cohort and we only included those with an available blood sample. This could have selected a group of preterm children with higher burden of morbidity if the parents were more prone to have extra follow-ups.

The strengths with paper II were the inclusion of healthy term controls when evaluating cellular aging in relation to prematurity and its longitudinal design. On the other hand, there might be a selection bias within the control group. Families of healthy children that consent to participate might have a heredity for certain diseases, for instance atopy, and thereby introduce a factor that may affect the results. Another difficult issue in research of newborn children is inclusion at birth. Preterm birth is rarely planned and it is difficult to get consent for research in a stressful time such as at birth. A limitation in paper II are the very few blood samples of preterm children at birth. Out of 197 available children for inclusion only 60 children, of which 16 were born preterm and 44 were born term, had longitudinal samples. It was a challenge to get samples from birth in the preterm group. On the other hand, the drop out for follow-up samples in the control group was much higher than in the preterm group.

Relevant for paper III were the same limitations with selection bias described above being part of a larger cohort study, but one strength was the inclusion frequency of more than 90% of available families. In studies including big data the problem with interference needs to be addressed.

The strength with an experimental study is the possibility to control for all factors. The biological relevance of findings in an animal model needs to be interpreted with caution before applicable to humans. One major limitation in paper IV was the protocol of ventilation that gave a short exposure to hyperoxia (one hour) and that was too short to find any detectable differences between groups treated with an antioxidant added to exogenous surfactant or not.

The considerations of study design are important due to the challenges of performing studies in extremely preterm infants early in life. Paper II and III had a longitudinal study design involving the exposure of neonatal intensive care. Extremely preterm born children can serve as a biological model of oxidative stress. However, the individual differences of both morbidity and the time of exposure of neonatal intensive care varies. Studies in humans are difficult to control because of the variations of individuals, but on the other hand the results will often be of biological relevance.

We study the exposure preterm birth and the obvious control group would be term born children with full health. Exposing healthy subjects for evaluation of lung function after mannitol and methacholine provocation were considered an ethical problem and therefore no healthy controls were included in paper I. Another problem with healthy controls is to get the consent of longitudinal blood sampling in children, particularly infants, and this was one of the challenges in paper II. Blood is a great resource for sampling. However, an extremely preterm infants has a total blood volume of 50 to 100 mL, compared to adults who has approximately 5 L, and it is difficult to longitudinally sample blood in extremely preterm children for research. The impact of environmental exposures postnatally and hereditary factors for consequences on immune development and cellular aging needs to be addressed. The study population of paper III was part of the TELLUS study. We have developed new blood sparing sampling methods that allowed us to perform comprehensive measurements

from small blood volumes, particularly important in preterm infants with small blood volumes. The resulting “neonate-omics”¹⁵⁵ permits improved assessments of global immune system data, biochemical and epigenetic data, and interactions in between.

The relevance of the findings

Telomeres and biological aging after prematurity

There seem to be a dynamic pattern of telomere length¹²² and telomerase activity¹³², but we still do not understand how this is regulated. We found longer telomere lengths in preterm born infants both at birth and at two years of age compared to healthy born term infants, which is consistent with others¹¹⁶⁻¹¹⁸. On the other hand, we found no difference in telomere length at 10 years of age due to prematurity (paper I), but that was a study without any healthy controls and of small sample size. Hadchouel et al found no difference in telomere length at 15 years of age in children born preterm or healthy term born¹¹⁵.

To our surprise, the telomere attrition rate was not accelerated in the preterm group during the first two years of life (paper II), and telomere length remained significantly longer in preterm infants at two years of age. From other studies we know telomere attrition rate plateaus at around four years of age¹²⁶, and maybe we have a very plastic time window from birth to four years of age where telomere length can be affected by different exposures such as inflammation and oxidative stress. Acute stress exposure led to an increased telomerase activity¹³² and the TERT subunit of telomerase protected mitochondria from oxidative stress¹³¹. Telomerase activity might counter-act accelerated telomere shortening during this time, and therefore no difference of telomere length due to prematurity in teenagers was found. Hadchouel et al¹¹⁵ found a correlation of shorter telomeres to impaired lung function, which was consistent to the findings in paper I¹.

The biological age of an individual can be estimated by DNA methylation analysis and we used the 353 CpG sites “epigenetic clock” prediction model described by Horvath^{138,139}. When comparing biological age to chronological age, preterm children were generally biologically younger than term children both at birth and at two years of age. This suggests that preterm children do not exhibit an accelerated cellular aging in spite of the susceptibility to oxidative stress damage and exposures to more severe viral infections during the first year of life. This might imply active repair mechanisms compensating for the stress of neonatal intensive care and the added stress following exposure to respiratory viral infections during the first year of life.

A major limitation to paper I and II are the lack of information of cell composition in the blood samples. The changes in telomere length and DNA methylation pattern can differ depending on cell composition. Wang et al found a great intra-individual variation in DNA methylation pattern during the first two years in life and they identified a pattern of methylation located in genes associated with biological functions including immunity and inflammation¹⁵⁶. De Goede et al showed that granulocytes and T-cells exhibit inter-

individual variability and differences in DNA methylation might not reflect prematurity but rather cell composition ¹⁵⁷.

The development of the preterm immune defense

The focus in paper III was to understand the developing neonatal immune system in the transition from intrauterine to extrauterine life. The preterm infants are born into a “cytokine storm”. Surprisingly the immune system of preterm and term infants rapidly converges onto a shared trajectory. That was surprising to us considering that extremely and very preterm infants were admitted to neonatal intensive care, sometimes for the whole study period, and term healthy newborns were discharged to home within 2-3 days of life. Analyses of the microbiome revealed that early gut bacterial dysbiosis could hamper the normal development. Arrieta et al proposed that the first 100 days in life were a critical period for the impact of microbial dysbiosis that was associated with the development of asthma ¹⁵⁸. For normal lung development a fine-tuned regulation seems to be required involving pathways such as the MAP kinases ¹⁵⁹ and inflammation can be part of disrupting this normal development ¹⁶⁰. Zasada et al described the genes involved in the development of the immune system of preterm infants during the first month of life (day 5 and 28) and concluded that despite differences in gestational age the pattern of gene expression was closely linked to postconceptional age. In the mucosal defense of newborn infants IgA is important ¹⁶¹ and are transmitted to the newborn infant by breast feeding. In the preterm infant genes important for host defense were activated, and most sensitive for gestational age differences were Ig A production in the intestine and signaling through the T-cells receptor pathway ¹⁶².

There are studies on the development of cell composition early in life and also the comparison of preterm compared to term children ^{157,163}. However, our study is the first study to prove a systems-level analysis across all cell populations present in the blood and that enables us to explain the interplay among all these different cell types early in life ¹³³.

Oxidative stress and lung function in experimental prematurity

Oxidative stress, inflammation and cellular aging are all involved in pathways important for cell survival, cell growth and cell death. If the cell survives it might be at a cost with triggered inflammation. Pathways involving regulation of NFκB, MAPK (p38) and HIFs are important pathways in preterm infants that are exposed to oxidative stress and inflammation, and make the link to senescence ^{164,165}. Some of these pathways are important for lung development ¹⁵⁹.

NFκB is one of the main regulators of early inflammatory responses. Hyperoxia will induce its activation and offer protection for the cell. NFκB play a physiological role in the developing lung and by regulating VEGF has an important role in both alveolarization and vascularization. Inhibited NFκB disrupted normal lung development in neonatal mice ¹⁶⁶ and suggests a mechanism contributing to the development of BPD. Different reactions to the NFκB receptor have been associated with different susceptibility to develop BPD, asthma and bronchiolitis ¹⁶⁰. TLRs are abundant in the airway epithelium and important to recognize

the action of pathogens and can modulate the response after lung injury of which activating NFκB is one. Surfactant protein A reduce cytokine expression and SP-D will inhibit the cytokine production of NFκB along with recruiting polymorphonuclear neutrophilic leukocytes by suppressing the TLR signaling ¹⁶⁷.

Great efforts have been put to try to prevent the development of BPD and the consequences of hyperoxia on lung impairment. Recombinant SOD given intratracheally increased the antioxidant capacity and decreased lung inflammation ¹⁶⁸ without benefits to respiratory outcome. N-acetylcysteine (NAC) given intravenously did not reduce BPD ^{169,170}. Natural surfactant has both antioxidative and immunological properties. Hyperoxia can inactivate surfactant and impair the antioxidant capacity.

In paper IV we used the antioxidant compound N-acetylcysteine amide in an experimental model of acute respiratory distress and hyperoxia in preterm rabbits. NACA is a precursor to glutathione, the most potent intracellular antioxidant, and have a good capacity to cross membranes. It has shown good results on protecting cells from oxidative damage, but also in several animal models protecting both the brain, kidney and eye ^{91,92,96,97,171-174}. NACA could prevent pulmonary inflammation by reducing infiltration of neutrophils and decreased the accumulation of ROS ¹⁷⁵. A reduction of ROS was seen in a mouse model of asthma where NACA also diminished inflammatory damage in the lung by regulating the NF-κB and hypoxia-inducible factor-1α (HIF-1α) activity ⁹⁵. Other antioxidants have been added to surfactant before ^{150,176-178}.

In our model the biophysical properties of porcine surfactant were preserved both *in vitro* and *in vivo* with different concentrations of NACA. The hyperoxia-induced surfactant inactivation could not be prevented by NACA. As a proof-of-concept, exogenous surfactant can serve as a vehicle for antioxidant treatment to the lung.

Treatments with an antioxidant needs to be considered carefully. The repercussion of very complex systems may interact with inflammation and immunological responses. It is necessary to find a biomarker that indicates which child should benefit of antioxidant treatment. Cellular aging is faster early in life compared to later in childhood or in adulthood and we still do not completely understand why. The results from our studies in understanding the consequences of environmental exposure after birth and the cellular mechanisms involved, suggests that the neonatal period in life can serve as a “window of opportunity” for treatments preventing oxidative stress and inflammation.

6 CONCLUSIONS

The immunological adaptation to extrauterine life are driven by microbial interactions and converge to a stereotypic pattern irrespectively of gestation. Preterm birth was not associated with increased biological aging (cellular aging), suggesting active repair mechanisms compensating for neonatal stressors. We need global analysis to understand the pathways and balances of the processes within the cells when exposed to microbes, inflammation and hyperoxia. A better understanding of this will help us identify infants with the need for treatments to prevent inflammation and cellular unbalances. Using exogenous surfactant as a vehicle to get an antioxidant for treatment of hyperoxia-induced lung damage locally might be feasible in the future.

Study I

Telomere length were similar in 10-year-old children born preterm with a history of BPD and term born children with allergic asthma in contrast to the proposed hypothesis. Impaired lung function with low forced expiratory capacity and male gender were associated with short telomeres irrespectively of preterm birth.

Study II

Children born preterm have early exposures to risk factors for accelerated cellular aging. Despite that, in contrary to the original hypothesis, preterm born children preserved telomeres and showed no accelerated epigenetic DNAm aging. This suggests the possibility of active repair mechanisms compensating for stress during the neonatal period.

Study III

Cord blood was not representative of postnatal immunity. The immune system of preterm and term children differed at birth but unexpectedly converged early in life and followed a stereotypic pattern of adaptation to extrauterine environment. This adaptation to microbial interactions of the environment may affect early immune system development.

Study IV

Hyperoxia impaired surfactant function in preterm surfactant-deficient rabbits, but this was not prevented by addition of the antioxidant N-Acetylcysteine amide. NACA did not affect the surfactant function and treatment effect. This is as a-proof-of-concept that exogenous surfactant can serve as a vehicle for antioxidants to the lung.

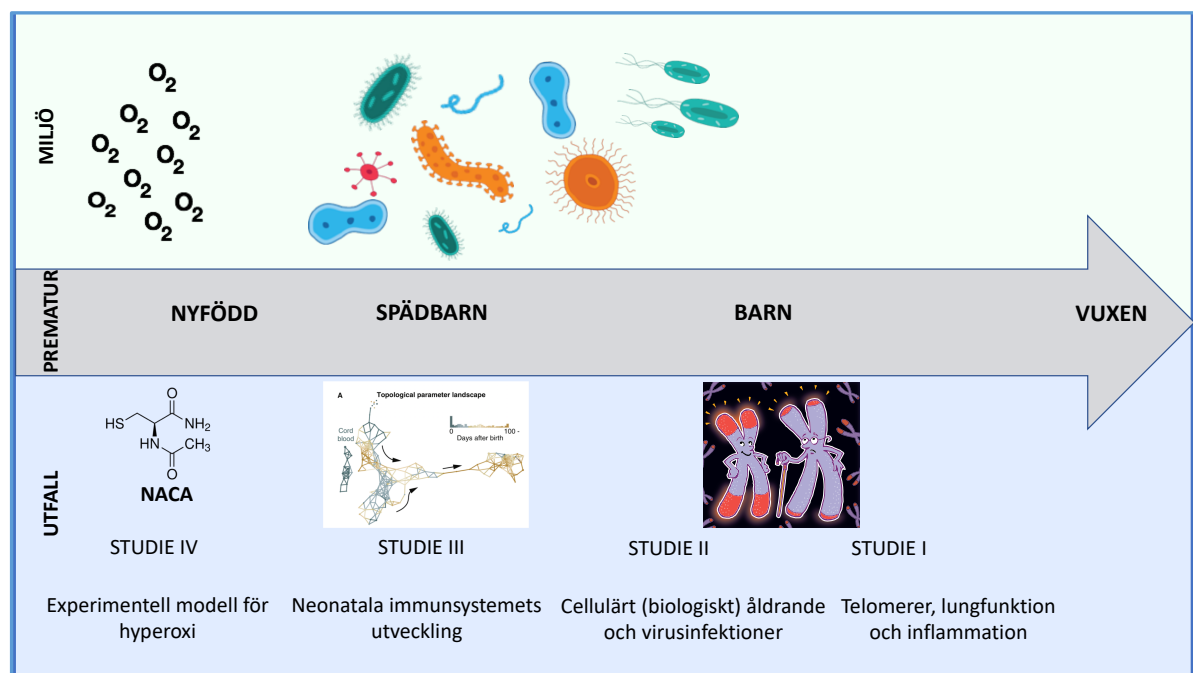
7 FUTURE PERSPECTIVES

This thesis will not have a direct impact on saving lives, it will try to understand the mechanisms of how preterm birth affects the adaptation to extrauterine life on a cellular level. The knowledge of interactions between inflammation and oxidative stress after preterm birth and the consequences of aging might facilitate the understanding of risk factors for age-related diseases and other inflammatory conditions. This will be important tools in the development of new medications and treatments. Preterm birth is emerging as an important risk factor for age-related diseases. Early preventions are necessary and the neonatal period might serve as an important “window-of-opportunity” to set the trajectory to cell survival without inflammation.

Systems-wide analysis provide us with the possibility to identify “panels” of pathways and processes in relation to cell composition involved in certain conditions, such as BPD. Analyzing the perinatal and morbidity data, together with food exposures and immunizations taking the hereditary aspects into account can create “panels”. These panels may in the future differentiate between healthy or medically compromised infants and serve as biomarker of certain conditions. In the future, a flow cytometry apparatus bedside at the neonatal intensive care unit might help the clinicians to decide whether a child needs antibiotics or not, or when the optimal time point for vaccination occurs to get the best immune response.

The development of blood sparing methods with a sampling protocol available around the clock and with facilities to prepare the samples at the neonatal intensive care unit was developed and described in paper III. One key issue has been the collaboration between clinical personal and researchers. We have this knowledge of how to use to systems-wide analysis using blood sparing protocols, the so called “neonate-omics”¹⁵⁵ and this can be systematically applied in other areas of interest and research.

8 SWEDISH SUMMERY – SVENSK SAMMANFATTNING



*Telomere picture
 behance.net/philtscheuer*

För tidigt födda barn (prematur) löper en stor risk att exponeras för oxidativ stress. Att lämna den skyddande miljön i livmodern för världen utanför, utgör en dramatisk miljöförändring för alla barn, men särskilt för barn födda prematurt. Foster utvecklas normalt i en relativt syrefattig miljö medan världen utanför är rik på syrgas. Skyddande antioxidativa system är omogna och både infektioner och behandlingar som prematurfödda barn behöver aktiverar inflammatoriska reaktioner. Vi förstår ännu inte de cellulära konsekvenserna av att födas för tidigt men oxidativ stress och inflammation spelar en nyckelroll.

I denna avhandling utforskar jag dessa fenomen hos prematurfödda barn. De fyra studierna kretsar kring olika aspekter av det för tidigt födda barnets anpassning till postnatal miljö. Vi studerar telomerlängd, lungfunktion och inflammation (studie I), cellulärt åldrande och virusinfektioner (studie II), det neonatala immunsystemets utveckling (studie III) och syrgas-inducerad lungskada och potentiering av surfaktant-skydd med hjälp av ett antioxidationsmedel (studie IV).

Vi har funnit att telomerlängden hos 10 åriga barn är jämförbar mellan barn födda för tidigt med utvecklad BPD under nyföddhetsperioden och fullgångna barn med utvecklade allergisk astma under barnaåren. En försämrad lungkapacitet och manligt kön var associerat till kortare telomerer oavsett gestationsålder (studie I). Mycket för tidigt födda barn bibehåller sina telomerlängder utan accelererat cellulärt åldrande (biologiskt åldrande) under de första två åren i livet (studie II). Immunförsvaret i navelsträngsblod förutsäger inte postnatala immunförsvaret på grund av dramatiska förändringar tidigt i livet. Dessa tidiga förändringar drivs av interaktioner mellan mikrober och följer ett stereotypt mönster i alla barn (studie III).

Syrgas-inducerad inaktivering av surfaktant i vår experimentella modell kan inte förhindras av en antioxidant, N-Acetylcysteine amide (NACA), men det påverkar inte heller funktionen av exogent tillfört surfaktant (studie IV).

I dessa studier har jag och mina kollegor utvecklat nya provtagningsmetoder för mycket små blodvolymmer vilket möjliggör longitudinella studier med upprepade provtagningar även hos de allra minsta barnen. De mest avancerade mätmetoderna kan nu kombineras till ett sorts, ”neonate-omics”, för global utvärdering av immunförsvaret och dess många olika signalvägar.

Med en bättre förståelse för det nyfödda barnets utmaningar när det föds mycket för tidigt, kan vi utveckla nya och mer individuella behandlingar samt anpassa vården, inte bara för att öka överlevnad, utan också överlevnad med framtida hälsa för våra allra minsta patienter.

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